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THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: Kimberly A. Gillis *et al.*

Application No.: 09/997,424 -- 6432

Filed: November 28, 2001

For: Expression Analysis of SMARC Nucleic Acids And
Polypeptides Useful In The Diagnosis And Treatment of
Prostate Cancer

Attorney Docket No.: 102729-16

Group Art Unit: 1642

Examiner: Minh Tam Davis

Certificate of Mailing (37 C.F.R. 1.8(a))

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Date of Signature and Mail Deposit	Thomas J. Engellenner Reg. No: 28,711

Commissioner for Patents
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DECLARATION OF STEVEN HANEY, PH.D.

PURSUANT TO 37 CFR §1.132

Dear Sir:

I, Steven Haney, a citizen of the United States residing at 72 Adams Road, Concord, MA 01742, hereby declare as follows:

1. I received a B.A. in biochemistry from the Stony Brook University, NY in 1984 and was awarded a Ph.D. in biochemistry from the University of Michigan in 1991. From 1991-1996, I was a Research Fellow at Princeton University, NJ. I am employed as a principal scientist in the Cancer Genetics Group at Wyeth located at 35 Cambridge Park Drive, Cambridge, MA 02140 and have been so employed since 2001. My responsibilities include conducting and supervising cancer genetic research, and in particular, I am involved in profiling gene expression in human prostate tumors in comparison to cancer cell line models. Prior to taking this

position in cancer genetics, I worked on genetic approaches to infectious diseases at Wyeth in Pearl River, NY for four years.

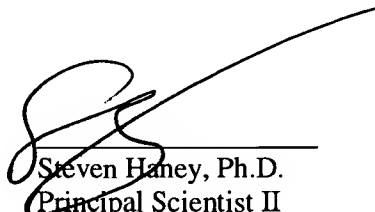
2. I am familiar with the above-identified patent application. I have reviewed the Office Actions mailed December 4, 2003 and July 15, 2004 with respect to the patent application at issue. I understand that the latest Office Action rejects the pending claims pursuant to 35 U.S.C. § 101 for lacking a specific, substantial or well established utility and pursuant to 35 U.S.C. § 112, first paragraph for lacking support by a specific, substantial or well-established utility. I have reviewed the application, and I believe that one skilled in the art would immediately appreciate that the invention has specific and substantial utility as a marker for human prostate cancer based upon the teachings of the application. I further believe that the disclosure in the application that expression of SMARCD-3 is decreased in the LNCaP cell line model of human prostate cancer provided support for this specific and substantial utility.
3. I understand that the Examiner has asserted that since change in the level of expression of a gene in a tumor as compared to normal corresponding cells is unpredictable, one cannot determine that SMARCD-3 would be differentially expressed in prostate cancer tissues versus corresponding normal cells.
4. Based on my knowledge and experience in the field, I believe and declare that one skilled in the art would recognize that the LNCaP cell line model used in the experiments described in the application is a well-characterized model of human prostate cancer. Consequently, a decrease in SMARCD-3 expression in the LNCaP cell line model is indeed predictive that expression would also be decreased in cancer tissue.
5. I understand that the Examiner has further asserted that the claimed method "is based on a flawed method, i.e. using Affymetrix screening an underrepresentative number of genes...[since]...the cRNAs from a total of 6000 genes of the claimed invention would not be representative of all mRNAs present in a cell." I have reviewed the application, and I believe that one skilled in the art would recognize that genes, which were found to be statistically significantly expressed ($p < 0.05$,

using a two-way analysis of variance (ANOVA)) in response to a natural androgen DHT in LNCaP cells based on the Affymetrix Genechip™ screening of 6800 full-length genes, provide specific and substantial utility as a marker for prostate cancer.

6. Well-characterized human cancer cell lines, such as LNCaP, are routinely used and have proven to be highly predictive of *in vivo* results. The LNCaP cell line, which was established from a metastatic lesion of human prostatic adenocarcinoma, has been widely used in the study of prostate cancer for over 20 years. The enclosed seminal journal article establishes the LNCaP cell line maintains malignant properties, hormonal responsiveness and drug sensitivity of the prostate adenocarcinoma (Horoszewicz, J.S. "LNCaP Model of Human Prostatic Carcinoma" *Cancer Research*, 43, 1809-1818 (1983)). Those skilled in the art view LNCaP cells as an established *in vitro* model of prostate cancer as evidenced by the fact that this seminal article establishing the LNCaP cell line has been cited over 800 times in other peer-reviewed journals.
7. Recent articles have verified that the LNCaP model of human prostate cancer progression and metastasis "closely mimics the genetic and pathological processes of cancer growth and progression in men" (Thalmann, G.N., et al. "LNCaP Progression Model of Human Prostate Cancer: Androgen-Independence and Osseous Metastasis" *The Prostate* 44: 91-103 (2000)). The LNCaP cell model expresses prostatic acid phosphatase, androgen receptor, and PSA, which is a hallmark of the prostatic phenotype and an invaluable marker of prostate cancer progression.
8. As described in the specification of the application at issue, the identified marker, SMARCD-3, was found to be significantly ($p < 0.05$) and differentially expressed between the diseased and normal tissues. The LNCaP well-established cell line model of human prostate cancer was used to show that SMARCD-3 decrease in expression in prostate cancer cells after androgen treatment. The prostate specific antigen (PSA) gene, which is recognized in the art as a diagnostic marker of prostate cancer, was used as an internal control showing that decreased expression of SMARCD-3 corresponds to the expected increase in PSA expression in prostate cancer cells.

9. Therefore, one skilled in the art would appreciate the accuracy with which the cell line model of prostate cancer mimics the genetics of human prostate cancer and further recognize the application's analytical techniques (e.g., RNA extraction, quantitative RT-PCR, western blot analysis, statistical analysis, and tissue microarray analysis) to be well established. One skilled in the art would conclude, as I have, that the *in vitro* data presented by the applicant is well correlated with the invention as claimed, namely the methods of the invention to diagnose or monitor development or progression of prostate cancer in humans.
10. I further declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: Oct 14, 2001


Steven Haney, Ph.D.
Principal Scientist II
Wyeth

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[CANCER RESEARCH 43, 1809-1818, April 1983]
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LNCaP Model of Human Prostatic Carcinoma¹

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ABSTRACT

The LNCaP cell line was established from a metastatic lesion of human prostatic adenocarcinoma. The LNCaP cells grow readily *in vitro* (up to 8×10^5 cells/sq cm; doubling time, 60 hr), form clones in semisolid media, are highly resistant to human fibroblast interferon, and show an aneuploid (modal number, 76 to 91) human male karyotype with several marker chromosomes. The malignant properties of LNCaP cells are maintained. Athymic nude mice develop tumors at the injection site (volume-doubling time, 86 hr). Functional differentiation is preserved; both cultures and tumor produce acid phosphatase. High-affinity specific androgen receptors are present in the cytosol and nuclear fractions of cells in culture and in tumors. Estrogen receptors are demonstrable in the cytosol. The model is hormonally responsive. *In vitro*, 5 α -dihydrotestosterone modulates cell growth and stimulates acid phosphatase production. *In vivo*, the frequency of tumor development and the mean time of tumor appearance are significantly different for either sex. Male mice develop tumors earlier and at a greater frequency than do females. Hormonal manipulations show that, regardless of sex, the frequency of tumor development correlates with serum androgen levels. The rate of the tumor growth, however, is independent of the gender or hormonal status of the host.

INTRODUCTION

CaP² is the second most frequent tumor of males in the United States (41). Unknown etiology, variable pathology, intricate relationship to endocrine factors, and anaplastic progression contribute to the complexity of this disease and confound the investigator. The progress toward establishing effective methods for early detection and successful management of CaP is predicated on both clinical studies and laboratory experimentation with appropriate models. Extensive studies on models of rodent prostate carcinoma (7, 9, 18, 23, 32, 40) have already provided a better understanding of the biology of prostatic neoplasia. These tumors, derived from rat prostate, are readily transplantable, and the tumor cells proliferate *in vitro*. Their value as models is based on the preservation of malignancy, biological and biochemical markers, hormonal responsiveness, and drug sensitivity. Their limitations stem from apparent restrictions imposed on

extrapolating data derived from animal models to human neoplasia.

The development of a correspondingly wide array of appropriate models of human origin is hindered by difficulties in sustaining the propagation of human CaP cells outside its natural host. With few exceptions (12, 33), the athymic nude mouse fails to provide satisfactory growth environment for direct CaP xenografts from patients (10, 34). Although human prostate cancer cells grow readily as short-term explant cultures (6, 16, 24, 45), reports on established malignant cell lines with convincing pedigree are rare (14, 20, 27, 42). In addition, some of such cell lines fail to maintain markers characteristic of CaP: e.g., production of secretory human prostatic acid phosphatase (19, 42); organ-specific prostate antigen (30); responsiveness to sex hormones (19, 42); or the presence of the Y chromosome (19).

The subject of this report is the cell line LNCaP which we have isolated from a metastatic lesion of human prostatic cancer (14). The growth properties *in vitro*, clonogenic potential, karyology, androgen responsiveness with its attendant presence of specific receptor molecules, and tumorigenicity in intact and hormonally manipulated athymic nude mice are described. These data extend our knowledge toward a full characterization of a versatile new model suitable to study human prostatic cancer in the laboratory.

MATERIALS AND METHODS

Culture Medium. The LNCaP cell line was originally established (14) and subsequently propagated in RPMI-1640-GA (Grand Island Biological Co., Grand Island, N. Y.) and supplemented with 5% (v/v) heat-inactivated single-lot (No. 300010H) FBS (K.C. Biological, Inc., Lenexa, Kans.). For hormone responsiveness experiments, ZSD (AMF Immune-Reagents Inc., Seguin, Texas) was utilized in place of FBS. ZSD is a commercially processed serum of bovine origin. The concentration of steroids is reduced by charcoal filtration by approximately 20-fold in comparison with regular FBS. Data provided by the manufacturer for the single lot (No. 050481) of serum used in our experiments indicated the following concentrations: testosterone, 72 pM; 17 β -estradiol, 58 pM; cortisol, not detectable; DHT, not determined; cholesterol, 0.52 μ M; insulin, 6 μ units/ml; and total protein, 45 mg/ml. The DHT was obtained from Sigma Chemical Co. (St. Louis, Mo.).

Cell Propagation. Confluent monolayers (6 to 8×10^5 cells/sq cm) of LNCaP cells cultivated in 75-sq cm plastic flasks (Falcon Plastics, Oxnard, Calif.) were dispersed with trypsin (0.05%):EDTA (0.02%) solution (Grand Island Biological Co.) and counted. The cells were inoculated into new vessels at 3 to 8×10^4 cells/sq cm in RPMI-1640-GA with 5% (v/v) FBS (0.1 ml/sq cm) and left undisturbed for 48 hr in the incubator (37 $^\circ$, 5% CO $_2$ atmosphere) to facilitate attachment. The cultures were fed at 6- to 7-day intervals with fresh medium (0.3 ml/sq cm) of the same composition. LNCaP cells from between the 30th to the 55th passage *in vitro* were used to conduct these studies. This corresponds to between 100 and 300 population doublings after the original isolation. The cells are free of *Mycoplasma*.

Cell Counts. An electronic particle counter (Model ZB-1 Coulter Counter) was used to enumerate formalin-fixed nuclei from detergent-

¹ This work was supported by USPHS Grant CA 27472 through the National Prostatic Cancer Project.

² To whom requests for reprints should be addressed.

³ The abbreviations used are: CaP, carcinoma of the prostate; RPMI-1640-GA, Roswell Park Memorial Institute Medium 1640 containing additional L-glutamine (1 mM) and antibiotics (penicillin, 100 units/ml; streptomycin, 100 μ g/ml); FBS, fetal bovine serum; ZSD, Zeta-Sera-D; DHT, 5 α -dihydrotestosterone; AP, acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2); HuIFN β , human interferon β (human fibroblast interferon); RPMI-1640, Roswell Park Memorial Institute Tissue Culture Medium 1640.

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lysed cells according to the method of Butler et al. (4).

Karyology. For cytogenetic analysis, LNCaP cultures were incubated with Colcemid (0.015 µg/ml; 2 hr; Grand Island Biological Co.), harvested, treated with hypotonic solution (0.075 M KCl), fixed with methanol:acetic acid (3:1), and spread on dry slides. The G-banding method (36) of staining was used for chromosome identification and analysis.

Sex Hormone Receptors. Methods used for the preparation of cytosols, nuclear fractions, and measurements of steroid receptors were published (11, 25, 39). Briefly, for androgen receptor determinations, the competition binding assay with methyltrienolone (17β-hydroxy-17-methylestra-4,9,11-trien-3-one) in the presence of triamcinolone acetonide was used (11, 43). Estrogen receptor was quantified by the dextran-coated charcoal assay (25) as well as the sucrose density gradient analysis (39) including nafoxidine as the competitor for the binding of radioactive 17β-estradiol.

Where appropriate, the binding was analyzed using the Scatchard plot method (38). The concentrations of protein (22) and DNA (3, 35) were determined as published.

[³H]Methyltrienolone (specific activity, 87 Ci/nmol) and nonradioactive methyltrienolone as well as 17β-[2,4,6,7-³H]estradiol (specific activity, 90 Ci/nmol) were purchased from New England Nuclear (Boston, Mass.). The trizma (free base) and triamcinolone acetonide were obtained from Sigma. Norit A charcoal was purchased from Matheson, Coleman and Bell Manufacturing Chemists (Norwood, Ohio); dextran (Grade C, M, 60,000 to 90,000) was obtained from Mann Research Laboratories (New York, N. Y.); and sucrose (RNase free) was purchased from Schwarz/Mann (Orangeburg, N. Y.). Dithiothreitol was obtained from Calbiochem (La Jolla, Calif.), and nafoxidine was a gift from the Upjohn Co., Kalamazoo, Mich.

AP. AP activity was determined by the method of Babson and Phillips (1) using 3 mM α-naphthyl acid phosphate sodium salt as the substrate, 0.02% Fast Red B salt (5-nitro-2-aminomethoxybenzene diazotate) as the color developer and 6 mM α-naphthol as the standard. All these reagents were obtained from Sigma.

HuIFNβ. The highly purified (specific activity, 2 × 10⁷ reference units/mg protein) HuIFNβ used in these studies was produced in our laboratory for clinical trials (21). Interferon assays were carried out using published methods (13, 15). The International reference standard of human fibroblast interferon (G-023-902-527; Research Resources Branch, NIH) was included in each assay for calibration, and titers were expressed in reference units as geometric means of triplicate tests.

Athymic Nude Mice. A breeding nude mouse colony has been maintained in our laboratory since 1976 under pathogen-limited conditions. The animals bear a nu/nu genotype on super Swiss and C3H background, and their life span is in excess of 12 months.

Tumor Induction. Tumors were induced in athymic nude mice (6 to 8 weeks old) by injection of cultured LNCaP cells. The cells were dispersed by trypsin, washed (twice) in serum-free medium RPMI-1640 (10 min centrifugation, 200 × g), resuspended (2 × 10⁷ cells/ml, same medium), and injected (0.2 ml) s.c. between the shoulder blades of each mouse. The tumors were measured with calipers (2 perpendicular diameters), and the tumor volume was calculated using the formula $W^2 \times L/2$ cu mm [W, shorter diameter; L, longer diameter (mm)].

Hormonal Manipulations. Castration or ovariectomy were performed 3 days before inoculation of LNCaP cells. Pellets, containing either 2 mg testosterone propionate or 2 mg 17β-estradiol, with cholesterol, Emcompress (Edward Mendel, Carmel, N. Y.), and magnesium stearate as excipients were prepared by the Roswell Park Memorial Institute Drug Formulation and Development Laboratory and implanted s.c. 3 days before inoculation of LNCaP cells. Both hormones were purchased from Sigma.

RESULTS

Studies *In Vitro*

LNCaP Cell Cultures. The LNCaP cells form monolayers which are only weakly attached to the surface of plastic culture

vessels. The cultures require gentle handling at all times because the cells could be easily dislodged by tapping, shaking, pipetting. The reattachment of dissociated cells is slow, and only about 70% of cells from the original inoculum adhered to the growth surface within 40 hr. Varying the concentration of FBS between 1 and 15% (v/v) does not measurably affect this process (data not shown). The low anchoring potential is also responsible for the 10 to 20% cell loss during media changes in long-term experiments. When cell density in excess of 8 × 10⁵ cells/sq cm is reached, the cell sheet tends to detach spontaneously. High concentrations of LNCaP cells, dispersed by routine trypsinization or by mechanical manipulations, form clumps which are difficult to dissociate or count reliably. Accurate cell counts can be obtained after lysing the cells and counting the free nuclei (4). To initiate growth in new cultures, a broad range of inocula (from 5 × 10³ to 1 × 10⁶ cells/sq cm) could be used (Chart 1).

Serum Requirement. Cultures of human cells *in vitro* require serum supplement for growth. The optimal concentration varies for different cells and is usually lower for cells derived from tumors than from normal tissues. To characterize further the LNCaP cells *in vitro*, their growth in RPMI-1640-GA supplemented with different concentrations of FBS [from 0.01 to 15% (v/v)] was measured. The results (Chart 2) show that LNCaP cells can proliferate in the presence of a broad range (0.1 to 15%) of FBS concentrations. Maximal growth was obtained using media supplemented with 2.5% to 15% FBS. Under these culture conditions, the mean population-doubling time was calculated to be approximately 60 hr. Lowering the serum concentration resulted in slower growth. At a FBS concentration of 0.01%, the number of cells in the culture slowly declined. For maintenance of cell stocks and experiments *in vitro*, 5% (v/v) FBS was routinely added to medium RPMI-1640-GA.

Cloning. Single malignant cells are capable of colonial growth in semisolid media. Table 1 shows that LNCaP cells have anchorage-independent proliferation and can be cloned easily and with good efficiency. From plates inoculated with the lowest number of cells, 13 individual clones were isolated, propagated, and cryopreserved (10% dimethyl sulfoxide, liquid N₂ storage) for future studies.

Karyology. The LNCaP cells were examined as to their karyological characteristics at 12 and 32 months after initial isolation, i.e., after approximately 100 and over 300 population doublings *in vitro*, respectively. At both points in time, the LNCaP cells

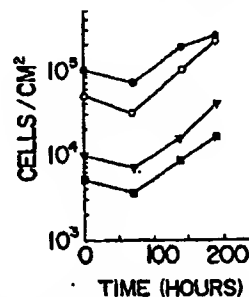


Chart 1. *In vitro* growth of LNCaP cells from inocula of different concentrations. Dispersed LNCaP cells were seeded in tissue culture multiwell plates (6 wells/plate; 9.82 sq cm/well) (Linbro, Hamden, Conn.) at 5 × 10⁵ (●), 1 × 10⁵ (○), 5 × 10⁴ (○), and 1 × 10⁴ (●) cells/sq cm and grown (37°, 5% CO₂) in RPMI-1640-GA with 5% (v/v) FBS. Cell counts (in triplicate from 3 wells) were carried out after 3, 6, and 8 days without media change. The number of cells per sq cm is plotted against time in hr after seeding.

exhibited grossly aneuploid human male karyotype with several marker chromosomes present. At 32 months, the number of chromosomes ranged from 33 to 91 with a modal number of 76

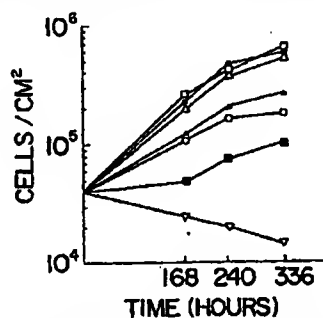


Chart 2. Growth of LNCaP cells *in vitro* in the presence of different concentrations of FBS. Dispersed LNCaP cells were seeded at 4×10^4 cells/sq cm in 8-well plates and grown (37°, 5% CO₂) in RPMI-1640-GA with different concentrations of FBS. Cell counts (in triplicate from 3 wells) were carried out after 7, 10, and 14 days without medium change. The number of cells/sq cm is plotted against time in hr after seeding. FBS concentrations (% v/v): ▽, 0.01; ■, 0.1; ○, 0.5; ▲, 1.0; △, 2.5; □, 7.5; ●, 15.

Table 1
Colonial growth of LNCaP cells in semisolid agar

Freshly trypsinized LNCaP cells (1.0 ml) in RPMI-1640-GA supplemented with 5% (v/v) FBS were mixed with an equal volume of the same medium containing 0.6% agar (40°). The dispersed cells were plated (in triplicate) over underlays (2.0 ml) of the same medium solidified with 0.5% agar in 50-mm plastic Petri dishes (Falcon Plastics). The plates were examined microscopically over a grid with 1-mm squares to count and to map any clumps containing 2 or more cells. After 3 weeks of incubation (37°, 5% CO₂), colonies derived from single cells were counted.

No. of cells/plate	% of total no. of cells plated	
	Cloning efficiency	Clumping rate
1×10^4	TMTC ^a	TMTC
1×10^5	TMTC	TMTC
5×10^5	15.0	3.8
1×10^6	12.0	1.8
5×10^6	10.4	0.8

^a TMTC, too many to count.

Table 2

Chromosome number distribution of LNCaP cells after 12 and 32 months of growth *in vitro*
The chromosome number was obtained from photographs of stained (trypsin-Giemsa) specimens of cells arrested in metaphase (see also Fig. 1).

Distribution of cells with following numbers of chromosomes																		Total No. of cells counted
	33	40	41	45	46	47	48	49	54	57	60	64	69	70	72	76-84 and above		
12 mos. <i>in vitro</i>					3	2	1		1	1		1				15	24	
32 mos. <i>in vitro</i>	1	2	1	4	4			1			2		1	2	2	26	46	

Table 3

Androgen and estrogen receptors in cultured LNCaP cells

For each of 2 experiments shown, 2×10^6 LNCaP cells were mechanically removed from culture vessels, centrifuged ($200 \times g$, 10 min, 0°), and washed twice in ice-cold serum-free RPMI-1640. The cell pellets were rapidly frozen and stored in liquid N₂ until used (within 30 days). Competition binding assays of cytosol and nuclear extracts by the dextran-coated charcoal procedure were used to determine androgen and estrogen receptor content. K_d values were derived from Scatchard plots. Protein was measured according to the method of Lowry *et al.* (22) and DNA according to Richards' modification (35) of the method of Burton (3).

Ex- peri- ment	Cells		Androgen receptor				Estrogen receptor (in cytosol)		
	mg DNA/g cells	mg cytosol protein/g cells	In cytosol		In nuclear extract		fmol/mg DNA	fmol/mg cyto- sol protein	K_d (nM)
			fmol/mg DNA	fmol/mg cyto- sol protein	K_d (nM)	(fmol/mg DNA)			
1	5.5	31.5	870	153	1.4 ^a	ND ^b	250	44	4.7
2	3.5	36.5	2160	266	0.9	309	585	72	5.2

^a For Scatchard plot, see Chart 3.

^b ND, not determined.

Human Prostatic Carcinoma Model

to 91 (in 26 of 48 metaphases counted) (Table 2; Fig. 1). Some marker chromosomes were present consistently: m_1 , long sub-metacentric marker chromosome of unknown origin; m_4 and m_6 , identified as t(1;15); m_5 and m_7 , identified as 10q-. The last 4 (m_4 to m_7) were usually present in 2 copies. Other chromosome abnormalities have been observed with lesser regularity.

Sex Hormone Receptors in Cultured LNCaP Cells. Specific androgen- and estrogen-binding proteins were reported in normal and neoplastic human prostatic tissues (8, 11, 28, 43). To determine if these macromolecules are present in the LNCaP cells maintained *in vitro*, androgen receptor in both the cytosol and the nuclear extract and estrogen receptor in the cytosol were measured. Table 3 shows that both specific androgen and estrogen receptors are present in the cytosol. Nuclear androgen receptor was also detected, but at a relatively lower concentration. Scatchard plot analysis (Chart 3) of the results indicated the presence of a separate single class of specific, high-affinity, low-capacity, and saturable androgen-binding molecules.

DHT Modulation of Cell Growth and AP Production. Human prostatic epithelial cells *in vivo* are responsive to male sex hormones. Therefore, both the cell proliferation and the AP production in LNCaP cultures in media supplemented with DHT were studied. The DHT concentrations tested (between 1 nM and 1 μ M) include and exceed the normal range of DHT levels found in the serum of healthy males (0.8 to 4.1 nM) (37). Simultaneous and parallel experiments were conducted using media supplemented with 5% (v/v) of either ZSD or FBS. ZSD was used because it is depleted of steroid hormones by activated charcoal during the manufacturing process. The LNCaP cells in RPMI-1640-GA supplemented with 5% (v/v) ZSD grew at a slower rate with a mean population-doubling time of approximately 144 hr as compared with the 60 hr for cells cultivated in medium with 5% (v/v) FBS. ZSD, however, does not contain apparent inhibitors of LNCaP cell division, since its addition (up to 10% v/v) to media already containing FBS (at 5% or 10% v/v) did not diminish the growth rate of the cultures (data not shown).

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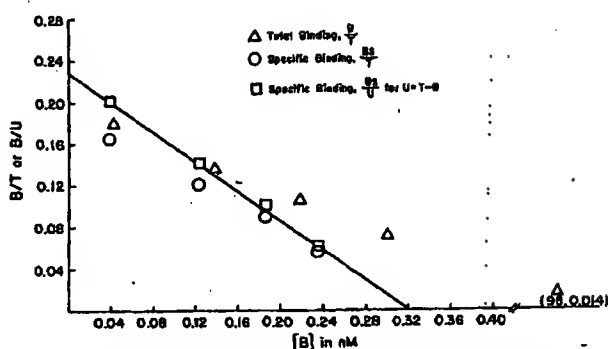


Chart 3. Scatchard plot analysis of androgen binding (B) in the cytosol from cultured LNCaP cells. Duplicate cytosol samples (200 μ l, 3.1 mg protein per ml) were incubated (22 hr, 4°) with [3 H]methyltrienolone (50 μ M, serial dilutions from 1 to 18.2 nM) in the presence and in the absence of nonradioactive methyltrienolone (508 nM). The mixtures contained also triamcinolone acetonide (1000-fold excess). Protein bound radioactivity was determined using the dextran-coated charcoal technique, and the fraction specifically bound was calculated. The calculated K_d is 1.4 nM.

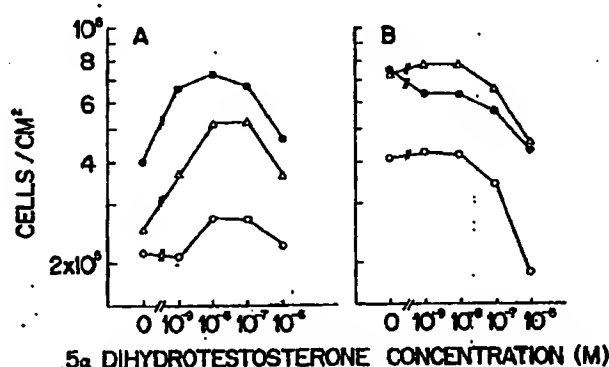


Chart 4. Effect of DHT on proliferation of LNCaP cells in culture. LNCaP cells were cultured for 20 days (37°, 5% CO₂) in medium RPMI-1640-GA with 5% (v/v) ZSD and then seeded in tissue culture 6-well plates (9.62 sq cm/plate) at 1×10^4 cells/sq cm in the same medium (0.1 ml/sq cm). Following attachment (48 hr), the medium was changed (0.2 ml/sq cm); one half of the cultures (A) were fed medium RPMI-1640-GA with 5% (v/v) ZSD, and the other half (B) were fed with 5% (v/v) FBS. At this time, DHT solutions (0.1 ml) in RPMI-1640-GA were added to each plate to obtain DHT concentrations indicated on the abscissa. Controls received 0.1 ml of a 0.2% solution of solvent mixture (ethanol:propylene glycol, 9:1) in which stock DHT solution (10 mM) was prepared, resulting in final ethanol concentrations of 0.07 mg/ml final propylene glycol concentrations of 0.01 mg/ml. Media and hormone were replaced after 5 and 8 days. Cell counts (in triplicate from 3 wells) were carried out at 120 hr (○), 192 hr (Δ), and 312 hr (●) after DHT addition. Media harvested at the time of cell counting were used to measure AP activity (see Chart 5).

The addition of DHT to growth medium has a distinct modulating influence on the proliferation of LNCaP cells (Chart 4). The orderly and dose-dependent curves obtained demonstrated 2 apparently different effects of DHT depending on which serum (ZSD or FBS) was present in the medium: (a) the addition of DHT to media supplemented with ZSD stimulated cell proliferation at all concentrations tested, with an optimum of about 10 nM (Chart 4A); and (b) DHT addition to media supplemented with FBS resulted in a dose-dependent suppression of cell proliferation (Chart 4B).

The AP activity in the LNCaP culture medium is also influenced by the addition of DHT. DHT was found to have potent stimulatory effects over the entire range of the tested concentrations

(Chart 5) regardless of which serum, ZSD or FBS, was used.

Resistance to Interferon. Interferons are glycoproteins with antiviral as well as antiproliferative activities. Both activities are readily demonstrable on a wide range of normal and neoplastic cell types. Since interferons are being tested as potential therapeutic agents in a variety of human tumors, including prostatic neoplasia, the *in vitro* susceptibility of LNCaP cells to HuIFN β was measured. HuIFN β , at concentrations as high as 10,000 reference units/ml, was found to have only a negligible effect on the proliferation of LNCaP cells during the 7-day period of exposure (Table 4). The presence of interferon did not affect the cell viability or influence the levels of AP production. The interferon levels detected at 24 and 96 hr postaddition (Table 4) indicate that the expected decay of interferon activity in LNCaP cultures is not greater than either the decay observed during incubation in cell-free medium (data not shown) or the decay reported for other cells (13).

To determine whether interferon could induce an antiviral state in LNCaP cells and thus prevent the killing action of vesicular stomatitis virus on these cells, HuIFN β (10 to 10,000 reference units/ml) was preincubated with the cells for 24 hr. Such treat-

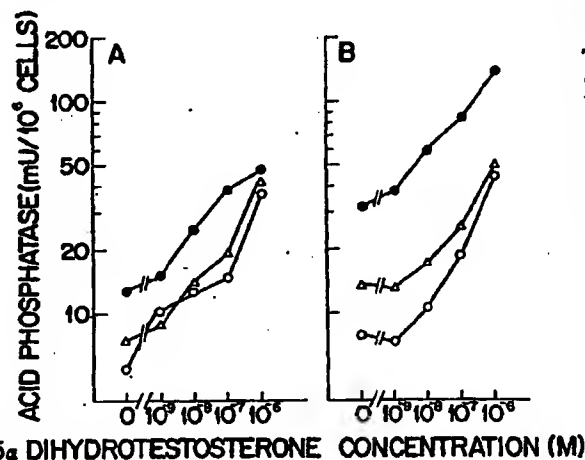


Chart 5. Effect of DHT on the activity of AP in LNCaP culture fluids. AP concentration was measured in triplicate in culture fluids from the same experiment illustrated by Chart 4. A, cells in RPMI-1640-GA with 5% (v/v) ZSD; B, cells in the same medium with 5% (v/v) FBS. Results are expressed in millunits (mU) of AP per 10^5 cells and are plotted against increasing concentrations of DHT on the abscissa 120 hr (○), 192 hr (Δ), and 312 hr (●) after DHT addition.

Table 4

Effect of HuIFN β on LNCaP cells

HuIFN β (0.1 ml) was added to LNCaP cultures (in quadruplicate, 5.8×10^4 cells/sq cm, 25-sq cm plastic flasks (Falcon Plastics) at Time 0. The growth medium (5.0 ml) and interferon were replaced at 96 hr. Total cell counts, viability, and AP activity in the culture fluids were determined at 168 hr. Interferon in culture fluids was assayed as published (13).

Interferon (reference units/ml) at			No. of population doublings at 168 hr	Cell viability (%)	AP (millunits/ 10^5 cells)
0 hr	24 hr	168 hr			
10,000	6,200	870	2.4542	81	5.4
1,000	790	45	2.7982	80	4.0
100	48	<5	2.8048	ND ^b	4.1
None	<5	<5	2.7993	83	4.5

^a By trypan blue exclusion.

^b ND, not determined.

Table 5

LNCaP tumors in male and female athymic nude mice

Mice (6 to 8 weeks old) were given s.c. injections of 4×10^6 cultured LNCaP cells and observed for 12 weeks. Tumors were measured with calipers at 5- to 7-day intervals. Data were combined from 2 to 6 experiments.

	Mean time of tumor appearance (days postinjection)	Tumor incidence (%)	Calculated mean tumor volume-doubling time (hr)
Males	$23.78 \pm 0.85^{a,b}$	58 ^c	85.5 ± 6.5^d
Females	33.84 ± 2.02^a	38 ^c	87.7 ± 5.1^d
Significance of difference	$p < 0.0001^e$	$p < 0.0005^e$	Not significant ^e

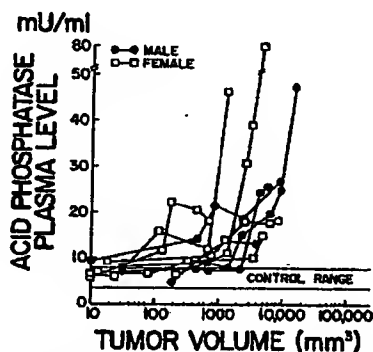
^a n = 28.^b Mean \pm S.E.^c n = 153.^d n = 15.^e n = 58.^f n = 162.^g Student's t test.^h χ^2 test.

Chart 6. AP activity in the plasma of athymic nude mice with LNCaP tumors. Plasma samples were taken at weekly intervals from 4 male and 5 female mice with LNCaP tumors. AP activity in the plasma was plotted against tumor volume at the time of sampling. Control range [4.1 to 9.4 mU/ml] was established on plasma samples from 11 male [6.46 \pm 0.54 mU/ml] and 9 female [6.09 \pm 0.56 mU/ml] athymic nude mice without tumors.

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ment did not result in protection of the cell sheet from total destruction (within 36 hr) when challenged with vesicular stomatitis virus at 100 times lower multiplicity of infection then used routinely for Interferon titrations. This result indicates that LNCaP cells in culture are at least 1 million-fold more resistant than are normal human diploid fibroblasts to the induction of antiviral protection by HuIFN β .

Studies in Athymic Nude Mice

Inoculum. The frequency of tumor formation following s.c. injection of 3.3×10^6 and 16.5×10^6 cells/animal did not significantly differ: 5 of 10 and 6 of 10 animals, respectively, developed tumors. No tumors grew after injection of 0.65×10^6 cells (10 mice) for up to 4 months of observation. For further studies, a tumor-inducing inoculum of 4×10^6 cells, injected s.c. in 0.2 ml, was chosen.

Tumor Formation and Growth. Nude mice given injections of LNCaP cells grown *in vitro* developed at the injection site poorly differentiated adenocarcinomas (14). Palpable tumors appeared between 14 and 56 days postinoculation. Once formed, the tumors grew rapidly (Chart 7). During the exponential phase of

Table 6

AP activity in the plasma and LNCaP tumors from nude mice

Four athymic nude mice with LNCaP tumors were bled, the plasma was collected, and AP activity was measured according to the method of Babson and Phillips (1). The tumors were dissected, weighed, and homogenized (ice bath; Sorvall Omni-Mixer with microattachment; 6000 rpm; 3 min) in acetate buffer (0.02 M; pH 5.0; 3 ml/g tumor) containing Tween 80 (0.01%). AP and protein were measured in the cytosol (supernatant after centrifugation of the homogenate at 105,000 g for 60 min) (1, 22).

Mouse	Sex	Tumor wt (g)	Plasma AP (mU/ml)	Cytosol AP	
				Total mU/ml	mU/ml/mg protein
1	Female	6.7	508	23,878	107
2		1.05	39	3,781	193
3	Male	5.4	18	12,115	34
4		6.4	26	15,577	39

Table 7

LNCaP tumor development in intact and hormonally manipulated athymic nude mice

Mice (6 to 8 weeks old) were castrated, ovariectomized, or implanted with hormone (2-mg) pellets 3 days before s.c. injection with 4×10^6 cultured LNCaP cells. Data from 2 experiments were combined: Experiment 1 involved Groups A, B, C, F, G, and H; Experiment 2 involved Groups A, B, D, E, F, G, I, and J (see also Chart 7).

Experimental groups	Plasma testosterone (ng/ml) ^a	Animals with tumors/animals inoculated	% with tumors	Statistically significant difference ^b at	
				$p < 0.01$ from Group	$p < 0.05$ from Group
Males					
A. Normal Controls	1.9	18/29	82	B, G	F
B. Castrated	<0.1	7/32	22	A, H	C
C. Castrated + testosterone ^c	0.45	8/14	57		B
D. Castrated + estradiol ^d	ND ^e	7/14	50		
E. Normal + estradiol ^d	ND	7/14	50		
Females					
F. Normal controls	<0.1	10/30	33	H	A
G. Ovariectomized	<0.1	7/26	26	A, H	
H. Ovariectomized + testosterone ^c	0.49	13/15	87	B, F, G	I
I. Ovariectomized + estradiol ^d	ND	6/14	43		H
J. Normal + estradiol ^d	ND	8/15	53		

^a Measured by radioimmunoassay (Bioscience Laboratories, Van Nuys, Calif.) on plasma samples pooled from 10 animals.

^b χ^2 test.^c Animals implanted with 2-mg testosterone propionate pellets.^d Animals implanted with 2-mg 17 β -estradiol pellets.^e ND, not determined.

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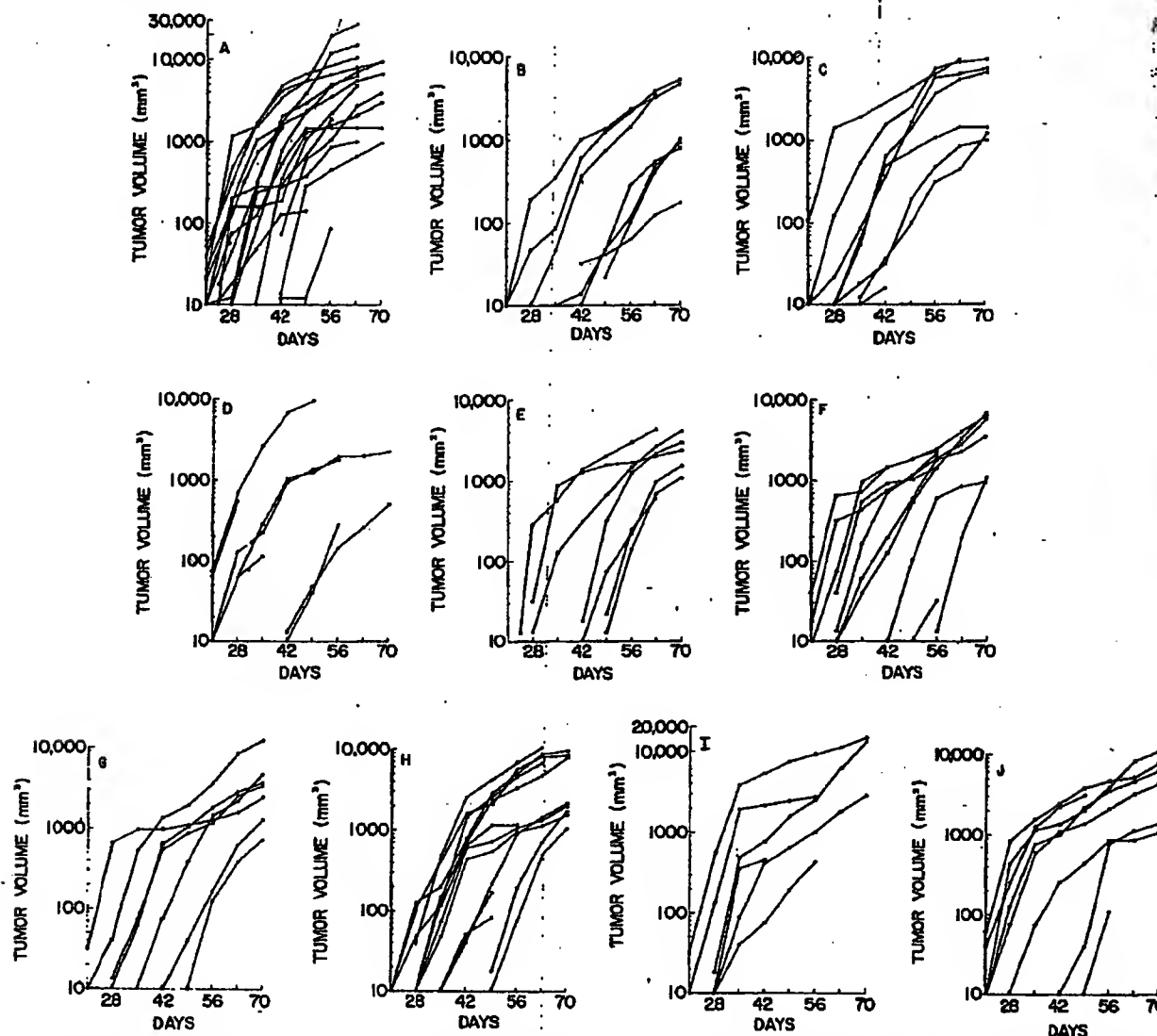


Chart 7. Kinetics of LNCaP tumor growth in intact and hormonally manipulated athymic nude mice. Experimental details were as described for Table 7. The tumor volume (ordinate) in cu mm was calculated from measurements of tumor diameters with calipers at the indicated times (abscissa) after injection of LNCaP cells. A, control intact males; B, castrated males; C, castrated males implanted with testosterone propionate (2-mg) pellets; D, castrated males implanted with 17β -estradiol (2-mg) pellets; E, males implanted with 17β -estradiol (2-mg) pellets; F, control intact females; G, ovariectomized females; H, ovariectomized females implanted with testosterone propionate (2-mg) pellets; I, ovariectomized females implanted with 17β -estradiol (2-mg) pellets; J, females implanted with 17β -estradiol (2-mg) pellets.

tumor enlargement, the calculated mean tumor volume-doubling time was about 86 hr (Table 5), which is only 1.5 times longer than the mean population-doubling time of LNCaP cells grown *in vitro*. Within 6 weeks after inoculation, the majority of tumors reached the weight of 1 g. The largest tumor observed weighed 28 g at 9 weeks postinjection. No apparent distal metastases were found upon autopsy and subsequent histological examination of the internal organs.

Tumors in Males versus Females. The frequency of tumor development and the mean time of tumor appearance are significantly different for males versus females (Table 5). Male mice develop tumors earlier and at a greater frequency than did females. The rate of tumor growth, however, is independent of

the gender of the host (Chart 7).

AP. Prostatic cancer in humans is frequently associated with elevation of prostatic acid phosphatase in the plasma. Since prostatic acid phosphatase is produced *in vitro* by LNCaP cells (14), the AP activity in LNCaP tumor cytosol and in the plasma of tumor-bearing animals was measured. In nude mice, the plasma AP level was found to increase concomitantly with the size of the tumors (Chart 6), but a considerable variation was encountered among individual animals. Ninety-six % of animals with tumors over 100 cu mm (33 of 35 males and 16 of 16 females) had plasma AP above 10 millunits/ml (*i.e.*, higher than the upper range in control mice); however, the magnitude of the plasma AP elevation did not reflect tumor volume on an individual

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basis. These observations could not be accounted for either by the total content of AP per tumor or by the relative AP activity per mg of cytosol protein. Table 6 shows that a 28-fold difference in plasma AP level was observed between 2 animals (Animals 1 and 3) while the total AP content per tumor differed only by 2-fold in these same animals.

LNCaP Tumors and Hormonal Manipulations. Prostatic cancer in humans, as well as animal models of prostatic neoplasia, have shown responses to hormonal manipulation of the host. In the LNCaP tumor model system, this was examined using 10 groups of athymic nude mice, each with a different hormonal status. Following injection of LNCaP cells, each group of animals was observed for time and incidence of tumor development (Table 7) as well as kinetics of tumor growth (Chart 7). Table 7 shows that, under conditions of androgen deficiency, the percentage of animals which developed tumors was significantly reduced. When the androgen deficiency was corrected by s.c. testosterone propionate implants, the tumor incidence returned to levels similar to those in intact males (Tables 5 and 7). Estrogen treatment did not result in a statistically significant modulation of tumor development. The graphic representations of individual tumor growth in each of the 10 experimental groups (Chart 7, A to J) illustrate that the rate of increase of tumor volume was essentially independent of the hormonal status of

the animals. These results demonstrate that the formation of LNCaP tumors is androgen responsive but that tumor growth is independent of intact gonadal function.

Sex Hormone Receptors in LNCaP Tumors. High-affinity specific androgen receptor (Chart 8) was found in the cytosol from all 38 nude mouse tumors examined (Table 8). No significant differences were observed in receptor content between tumors from intact and hormonally manipulated animals of both sexes (Table 8), regardless of whether the results are expressed as per g of tissue, per mg protein, or per mg DNA. No correlation emerged between the tumor size and the cytosol androgen receptor concentration (Chart 9).

Although the concentration of the nuclear androgen receptor was low [118 ± 23 (S.E.) fmol/mg DNA and 453 ± 92 fmol/g of tumor], it was detected in 12 of 38 tumors examined. Nonquantifiable trace amounts of nuclear androgen receptor were found in 20 additional tumors. In the remaining 6 tumors, no nuclear androgen receptor was detected.

Estrogen receptor (Table 9) was present in the cytosol from all 14 tumors examined from nude mice (intact, castrated, or ovariectomized). The specific and high-affinity (K_d from 1.2 to 5.5 nM) macromolecules sedimented in the 8S region using the sucrose density gradient centrifugation procedure.

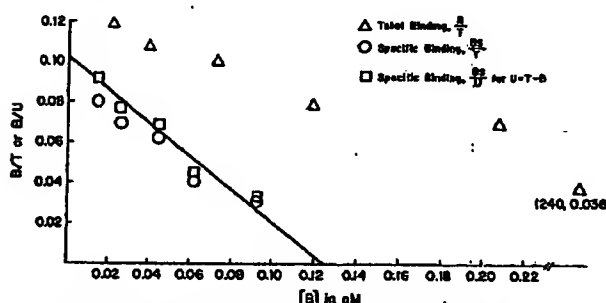


Chart 8. Scatchard plot analysis of androgen binding (B) in the cytosol prepared from a LNCaP tumor in a male athymic nude mouse. Duplicate cytosol samples (200 μ l, 2.3 mg protein per ml) were incubated (22 hr, 4°C) with [3 H]methyltrienolone (50 μ l, serial dilutions from 1 to 16.2 nM) in the presence and in the absence of nonradioactive methyltrienolone (508 nM). The mixtures contained also triamcinolone acetonide in 1000-fold excess. Protein bound radioactivity was determined using the dextran-coated charcoal technique, and the fraction specifically bound was calculated. The calculated K_d is 1.2 nM.

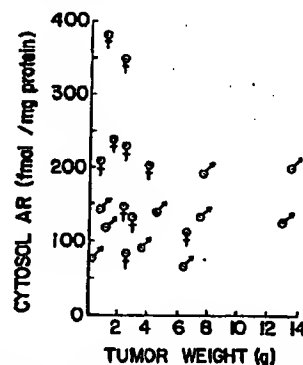


Chart 9. Cytosol androgen receptor (AR) in LNCaP tumors of different weights. Tumors induced in male and female athymic nude mice after injection of cultured LNCaP cells were removed at 3 to 10 weeks postinoculation and weighed (abscissa). The concentration of the androgen receptor in the cytosol was measured according to the method of Hicks and Walsh (11); protein was measured according to the method of Lowry et al. (22) (ordinate).

Table 8

Cytosol androgen receptor in LNCaP tumors from athymic nude mice

Tumors were induced by s.c. injection of 4×10^6 cultured LNCaP cells in 6- to 8-week-old nude mice. Ovariectomy, castration and s.c. implants with 2-mg testosterone propionate pellets were performed 3 days before injection of LNCaP cells. Ten weeks after inoculation, the tumors were removed and stored in liquid N_2 . Androgen receptor in the cytosol prepared from individual tumors was measured by the competition binding assay with methyltrienolone according to the method of Hicks and Walsh (11). Protein was measured according to the method of Lowry et al. (22); DNA was measured according to Richards' modification (35) of the method of Burton (3).

Tumor host	No. of tumors	Cytosol protein content (mg/g tumor)	DNA content (mg/g tumor)	Cytosol androgen receptor concentration			K_d (nM)
				fmol/mg protein	fmol/mg DNA	fmol/g tumor	
Male	10	50.8 ± 5.3^a	4.2 ± 0.37	129 ± 16^b	$1,682 \pm 236$	$6,965 \pm 1,227$	1.04 ± 0.16
Castrated male	4	60.9 ± 1.3	4.0 ± 0.05	126 ± 16	$1,930 \pm 240$	$7,683 \pm 875$	0.85 ± 0.03
Castrated male with testosterone implant	5	52.0 ± 3.6	3.3 ± 0.19	117 ± 12	$1,844 \pm 144$	$6,054 \pm 631$	0.71 ± 0.04
Female	10	52.7 ± 4.2	4.6 ± 0.33	208 ± 31^b	$2,709 \pm 818$	$11,198 \pm 2,245$	1.44 ± 0.22
Ovariectomized female	2	77.1	4.5	194	3,320	14,880	0.80
Female		58.0	4.4	125	1,590	7,000	0.60
Ovariectomized female with testosterone implant	7	40.8 ± 1.6	3.1 ± 0.16	177 ± 21	$2,284 \pm 212$	$7,171 \pm 820$	0.85 ± 0.004

^a Mean \pm S.E.

^b Significant difference analyzed by Student's *t* test at $0.05 > p > 0.02$. None of the remaining differences between groups is significant at $p < 0.05$.

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Table 9

Cytosol estrogen receptors in LNCaP tumors from athymic nude mice

Tumors were induced by s.c. injection of 4×10^6 cultured LNCaP cells in 8- to 8-week-old nude mice. Ovariectomy or castration was performed 3 days before injection of LNCaP cells. Ten weeks after inoculation, the tumors were removed and stored in liquid N_2 . Estrogen receptor in the cytosol prepared from individual tumors was measured both by the competition binding assay with DCC* (25) and by the SDGC analysis (39) with nafoxidine as the competitor.

Receptor concentration (fmol/mg cytosol protein)							
Males				Females			
Intact		Castrated		Intact		Ovariectomized	
SDGC	DCC	SDGC	DCC	SDGC	DCC	SDGC	DCC
1.8	7.7	7.0	51.3	5.2	34.4	ND	2.6
2.7	14.3	ND	14.8	5.9	60.2	ND	6.9
2.0	21.0	ND	14.4	4.8	26.5		
4.9	22.9		19.5	2.8	23.3		

* DCC, dextran-coated charcoal after 18 hr incubation; SDGC, sucrose density gradient centrifugation after 4-hr incubation (8S peak); ND, not determined.

DISCUSSION

This report characterizes the cell line LNCaP and demonstrates its utility as a model for laboratory studies on human prostatic cancer *in vitro* and in the athymic nude mice.

The LNCaP cells established from human CaP (14) could be readily propagated in the laboratory by routine cell culture methods if appropriate measures are taken to prevent the weakly attached cells from being dislodged from the plastic growth surface. High growth saturation densities of monolayers, adequacy of low serum concentrations to promote cell division, anchorage-independent proliferation in semisolid media, and excellent cloning efficiency of the LNCaP cells are consistent with the generally recognized properties of neoplastic cells *in vitro*.

The LNCaP cells are aneuploid. They have a full complement of human chromosomes, including the Y-chromosome as well as several marker chromosomes. Their karyotype is clearly different from that of HeLa cells which in the past were frequently mistaken for some established malignant cell lines (29). The continuously maintained high degree of polyploidy, as well as the major karyological characteristics in LNCaP cultures, examined at 20 months or approximately 200 population doublings apart, suggest that the selection pressure from growth conditions *in vitro* has not eliminated the apparent heterogeneity of the LNCaP cells.

Evidence that the LNCaP cell line originated from human prostatic cancer tissue is provided by: their morphology (14); preservation of functional differentiation; and maintenance of malignant properties in the athymic nude mice. Organ-specific glycoproteins, such as human prostatic acid phosphatase (14) and prostatic antigen (30), are present in cultured cells, in cell culture fluids, in tumors induced in the nude mice, and in the plasma of tumor-bearing animals.

Functional differentiation, consistent with human prostatic epithelial derivation, is also reflected by the responsiveness of the LNCaP cells to androgens. The cellular proliferation *in vitro* is modulated in a dose-dependent manner by the presence of DHT in the culture medium. In addition, DHT has an enhancing influence on AP production by the LNCaP cultures. This effect is apparently independent of the mitotic stimulation induced by DHT. Under conditions when DHT suppresses cell growth, the level of secretory AP in medium continues to rise. These observations suggest that the influence of androgen on cell division and AP synthesis may follow different regulatory pathways in

human CaP. Recently, Hudson (16) reported an increase in the level of AP when short-term primary cultures of human CaP were maintained in media with DHT or testosterone. The detection of a specific, high-affinity, low-capacity, saturable androgen receptor in the cytosol and nuclear extracts from both cultured LNCaP cells, as well as in nude mice tumors, provides the molecular basis for the observed androgen responsiveness *in vitro* and *in vivo*. LNCaP cells also contain specific estrogen receptor in the cytosol, a finding consistent with the previously described presence of estrogen receptor in normal and malignant human prostatic epithelium (28).

The presence of complete FBS in culture medium was found to prevent the demonstration of DHT growth-stimulatory effects in LNCaP cells. Such effects became apparent when steroid-depleted, charcoal-treated serum, e.g., ZSD, is used as the growth medium supplement. The reasons for this are unclear, but these findings imply the existence of a significant interplay between androgens and charcoal-removable serum components in the regulation of CaP cell proliferation. Several conflicting observations (19, 24, 42, 44) on the effects of androgens on the *in vitro* growth of normal and malignant human prostatic epithelium are perhaps due, in part, to the use of a variety of complete mammalian sera as medium supplement.

Malignant properties and hormonal responsiveness *in vivo* of the LNCaP cells are maintained. After injection of the uncultured LNCaP cells into nude mice, the time and frequency of tumor development is favored by androgens. However, once the tumors are established, their growth rates are similar, regardless of the gender or hormonal manipulation of the animals suggesting that the LNCaP cultures may consist of cells which are heterogeneous in their responsiveness to sex hormones. This is supported by published evidence (17) that in the castrated rats the progression of the Dunning prostatic adenocarcinoma to an androgen-independent state is due to the basic heterogeneity of the original inoculum and a rapid selection process *in vivo*. Future experiments with single-cell-derived clones could resolve this issue.

The resistance of the LNCaP cell lines to the antiproliferative as well as the antiviral effects of HuIFN β implies, but does not prove conclusively, the existence of a defect in the cell receptor for type I Interferon (2). Such defects are rare among human cells (5). It may therefore be of value to assess, before large-scale clinical trials with interferon are undertaken in this disease, whether LNCaP cells are unique in this respect or whether Interferon resistance is a common property of human CaP.

Recent observations (unpublished) in our laboratory on the sensitivity of LNCaP tumors in nude mice to chemotherapeutic regimens currently used in the treatment of CaP suggest the probable utility of this model for screening new drugs or combinations of drugs for potential activity against prostatic neoplasia.

The complexity of CaP requires several model systems available to the investigator to study this disease (26). The well-characterized LNCaP cells should find its proper place in research aimed at finding relevant answers concerning etiology, genetic stability, hormonal regulation, immunological properties, and the detection (30, 31) and therapy of human prostatic carcinoma.

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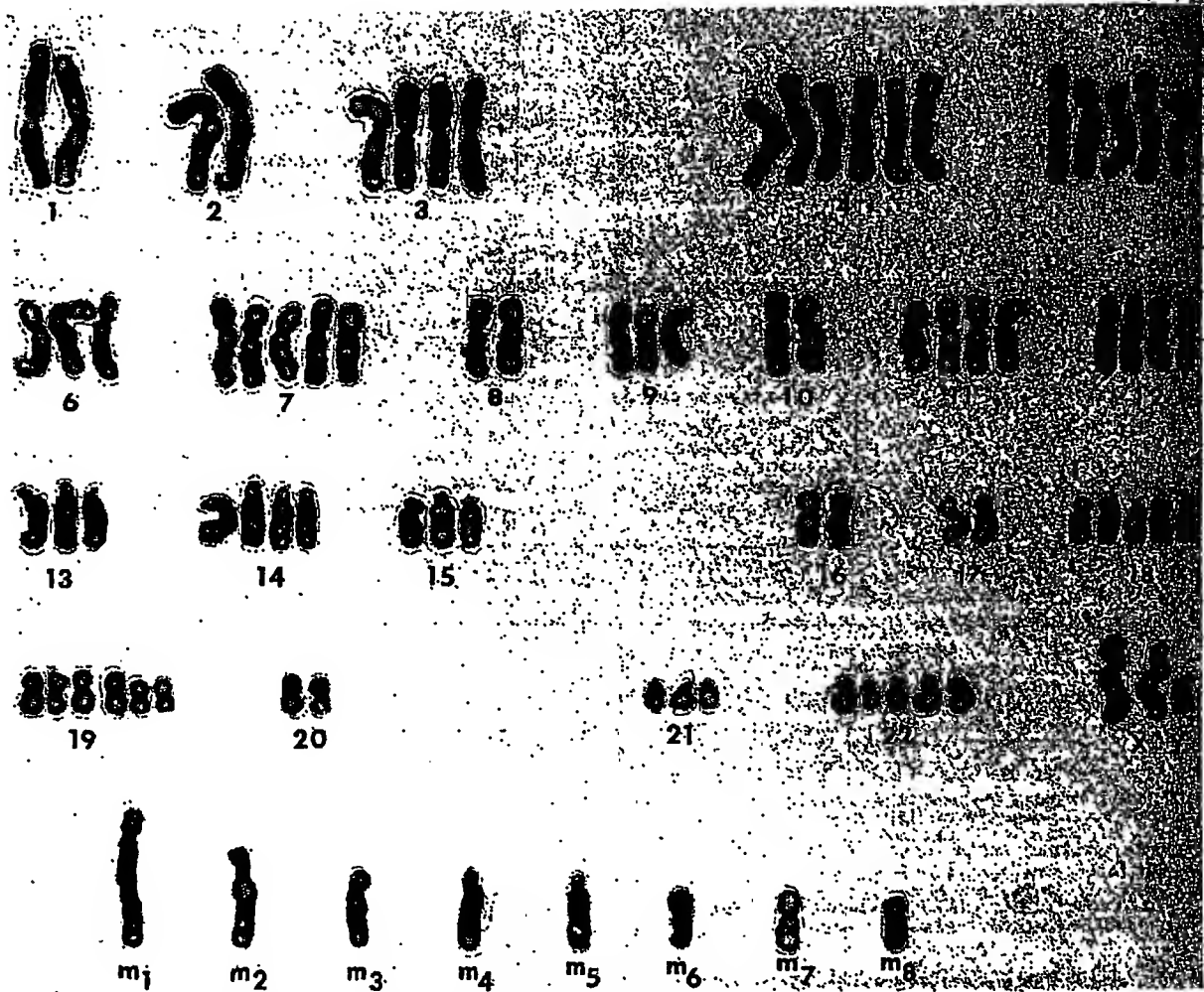


Fig. 1. G-banded karyotype of a LNCaP cell from a culture maintained for 32 months *in vitro* after isolation. One set of normal human chromosomes is present in addition to multiple copies and marker chromosomes (m₁ to m₈) for a total of 88 chromosomes.

LNCaP Progression Model of Human Prostate Cancer: Androgen-Independence and Osseous Metastasis

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BACKGROUND. Clinically, the lethal phenotypes of human prostate cancer are characterized by their progression to androgen-independence and their propensity to form osseous metastases. We reported previously on the establishment of androgen-independent (AI) human prostate cancer cell lines derived from androgen-dependent (AD) LNCaP cells, with androgen independence defined as the capability of prostate cancer cells to grow in castrated hosts. One of the sublines, C4-2, was found to be AI, highly tumorigenic, and metastatic, having a proclivity for metastasis to the bone.

METHODS. We established the AI and bone metastatic cell sublines B2, B3, B4, and B5 from the parental C4-2 subline, using a previously established coinoculating procedure. We determined the biologic behavior of the parental and derivative LNCaP sublines in vivo and in vitro, as well as their molecular and cytogenetic characteristics.

RESULTS. Unlike other human prostate cancer models, the LNCaP progression model shares remarkable similarities with human prostate cancer. We observed a comparable pattern of metastasis from the primary to the lymph node and to the axial skeleton, with a predominant phenotype of osteoblastic reaction; 25–37.5% of the animals developed paraplegia. Cytogenetic and biochemical characterizations of LNCaP sublines also indicate close similarities between human prostate cancer and the LNCaP progression model. Additional chromosomal changes were detected in B2–B5 sublines derived from C4-2 bone metastases. These LNCaP sublines were found to grow faster under anchorage-dependent but not -independent conditions. The in vitro invasion and in vivo metastatic potential of these LNCaP sublines surprisingly correlated with anchorage-dependent and not -independent growth. The derivative LNCaP sublines when cultured in vitro produced a substantially higher (20–30-fold) amount of basal steady-state concentrations of PSA than that of the parental LNCaP cells. PSA production was high initially, but was markedly reduced when the derivative cell lines were inoculated and allowed to grow long-term in vivo for the establishment of tumors and metastasis, suggesting that unknown host factors derived either from the prostate or the bone can effectively downregulate PSA expression by prostate tumor epithelium.

CONCLUSIONS. The LNCaP model of human prostate cancer progression will help improve

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our understanding of the mechanisms of androgen-independence and osseous metastasis, and tumor-host determinants of PSA expression. *Prostate* 44:91–103, 2000.

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KEY WORDS: prostate cancer progression; androgen-independence; stromal-epithelial interaction; skeletal metastasis; PSA expression; cytogenetics; CGH; chromosomal losses and gains

INTRODUCTION

The last decade has brought increased attention to and awareness of prostate cancer as a significant public health problem [1]. Prostate cancer is recorded as the leading cancer diagnosed and the second cause of cancer death in North American men [2]. Observations at autopsy indicate that early prostate cancer evolves in a multifocal pattern within the gland. Although the treatment of localized disease has significantly improved, once prostate cancer progresses to the periprostatic space by penetration and perforation of the prostate capsule and/or by invasion of the perineural spaces to the lymph nodes, few therapeutic options with limited durability are available. Clinical evidence suggests that after an initial responsiveness to androgen withdrawal, prostate cancer relapses to an androgen-independent state and metastasizes nonrandomly to the bone.

Little is known about the biology of prostate cancer metastasis, the underlying mechanisms of metastasis and androgen-independence, and the reciprocal interactions between prostate cancer epithelial cells and their surrounding stromal cells. This is due mainly to the fact that few *in vivo* model systems exist that closely mimic the natural history of progressive and metastatic human prostate cancer. By means of constructing a cell-cell recombination model [3,4], our laboratory has observed the growth and androgen-independent progression in a human LNCaP prostate cancer model. A series of lineage-related LNCaP cell sublines that reflect the various steps of prostate carcinogenesis and progression has been derived [5,6]. An androgen-independent (AI) cell line, C4-2, reproducibly and consistently follows the metastatic patterns of hormone-refractory prostate cancer by producing lymph node and bone metastases when injected either s.c. or orthotopically in either hormonally intact or castrated hosts [5,6]. This model permits the study of factors that determine the tropism of prostate cancer cells for the skeletal microenvironment.

The goals of the present study are twofold. First, we wish to establish rapid bone metastatic LNCaP human prostate cancer sublines from an animal model of human prostate cancer skeletal metastasis for future mechanistic and therapeutic studies. Second, we wish

to characterize these LNCaP sublines with respect to their biologic, cytogenic, and biochemical characteristics. Our findings indicate that: 1) Stromal cells and host endocrine status play a pivotal role in "selecting" or "inducing" prostate cancer cells to acquire androgen-independence and osseous metastatic potential. 2) Specific cytogenetic alterations are associated with LNCaP sublines as a result of androgen-independence and metastatic progression. 3) Anchorage-dependent rather than -independent growth of the LNCaP sublines correlated with prostate cancer invasion *in vitro* and their metastatic potential *in vivo*. 4) PSA expression by LNCaP sublines appears to be dysregulated and is independent of the presence of androgen but is highly sensitive to regulation by undefined host factor(s). The LNCaP prostate cancer progression model reveals a basic principle in which epigenetic factor(s) derived from the host can "drive" the progression of a human prostate cancer cell line, LNCaP, to androgen-independence as well as to acquire osseous metastatic potential with defined cytogenetic changes.

MATERIALS AND METHODS

Establishment of an *In Vivo* Human Prostate Cancer Model

LNCaP cells, passage 29 of the original line developed by Horoszewicz et al. [7], were kindly supplied by Dr. Gary Miller (University of Colorado, Denver, CO). Passages 25–33 of a human bone fibroblast cell line, MS, established from a patient with an osteogenic sarcoma as described previously [8], were used in this study.

Six- to eight-week old athymic nude mice (ncr strain [Balb/c background]), obtained from Charles River Laboratories (Baltimore, MD), were used for all *in vivo* experiments. They were kept under pathogen-free conditions in laminar flow boxes in accordance with established institutional guidelines and approved protocols. Unless otherwise specified, LNCaP tumors were induced by s.c. coinjection of 1×10^6 LNCaP cells and 1×10^6 MS cells into male athymic nude mice, as described previously [8]. Typically, cells were inoculated s.c. at 2–6 sites into the flank of intact mice. After 8 weeks, some mice were castrated bilaterally by scrotal incision under methoxyflurane

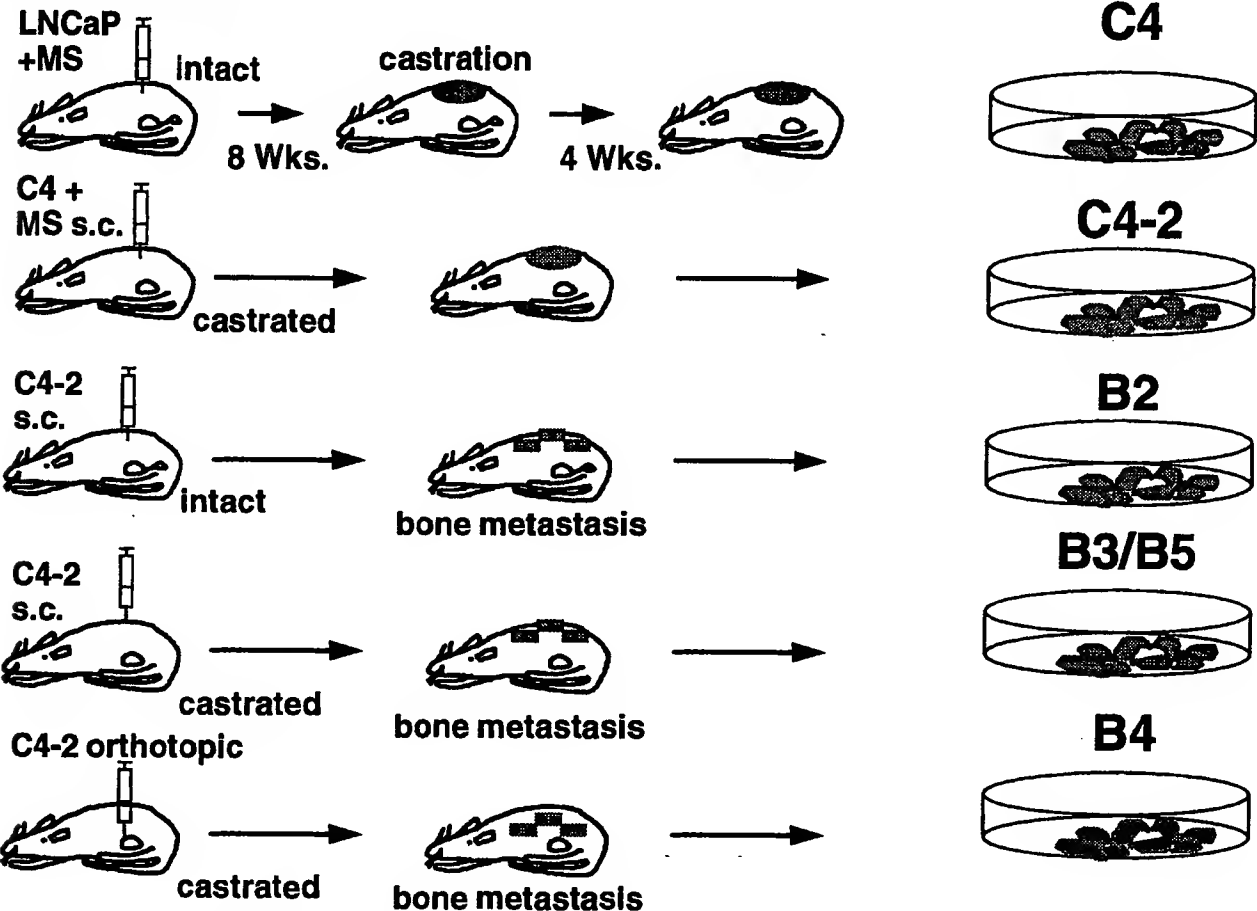
Co-Inoculation s.c.

Fig. 1. Schematic derivation of LNCaP sublines from tumors maintained in intact and castrated athymic male mice. MS, fibroblasts derived from a human osteosarcoma.

(Metofane®) anesthesia, and others were sham-operated. The tumors were maintained in the castrated hosts for an additional 4 or 5 weeks (Fig. 1).

The C4 and C5 LNCaP sublines were established from tumors harvested from the castrated hosts at 4 weeks (C4 subline) and 5 weeks (C5 subline) postcastration, as described [8]. A control LNCaP subline (subline M) was established from a LNCaP tumor maintained in an intact male host for 12 weeks (a sham operation was performed at 8 weeks), as described [8]. The C4-2 subline was established in a similar manner from a C4/MS chimeric tumor grown in a castrated male host [5]. All cell lines, passages 23–35, were grown in T-medium as described [9]. The cells were tested and found free of *Mycoplasma*.

For subcutaneous injection of C4-2 cells (1.0×10^6 cells/site), the cells were resuspended in RPMI-1640 and 10% FBS and injected in 0.1 ml/site (27-gauge needle, 1-ml disposable syringe) at 6 sites per mouse. For orthotopic administration of the tumor cells ($1.0 \times$

10^6 cells), the cells were resuspended in the same medium (total volume of 20 μ l) and were delivered to the dorsolateral lobe of the prostate gland of athymic mice by a 30-gauge needle, using a calibrated push-button Hamilton syringe (Reno, NV). Orthotopic injections were performed under methoxyflurane (Metofane®) anesthesia, with the prostate lobe exposed following lower midline abdominal incision. The wound was closed by metal clips (Autoclip, Clay Adams, Parsippany, NJ).

Establishment of Fast-Growing Human Bone Metastatic Cell Lines

In order to study the *in vivo* behavior of bone metastasis, we derived fast-growing sublines B2–B5 derived from bone metastases that have a defined cell lineage relationship with C4-2 (Fig. 1). C4-2 tumor cells, which adhere more loosely to culture dishes than fibroblasts and osteoblasts, were recovered by me-

chanically irrigating these cells with tissue culture medium. Pure bone metastasis-derived sublines B2–B5, as judged by morphological, cytogenetic, and immunohistochemical criteria, were obtained after 8–12 rounds of these subculturing steps.

The bone metastasis-derived sublines B2–B5 were grown in T-medium (see above) with 5% FBS. The cells were routinely tested and found to be free of *Mycoplasma*. Unless indicated otherwise four mice were injected (27-gauge needle, 1-ml disposable syringe) subcutaneously with 1.0×10^6 B2, B3 (8 mice), B4, and B5 cells resuspended in 0.1 ml of T-medium and 10% FBS at 4 sites per animal. (The mice were bilaterally castrated by scrotal incision under methoxyflurane (Metofane®) anesthesia. The wound was closed by metal clips. Animals were routinely inspected for physical abnormalities and tumor growth. Tumors were measured weekly, and their volumes were calculated by the formula $L \times W \times H \times 0.5236$ [10].

Characterization of the Parental and LNCaP Sublines: Crystal Violet Growth Assay

Cellular growth rates for LNCaP, C4-2, and B2–B5 sublines were determined using a 96-well plate (Becton Dickinson, Lincoln Park, NJ) assay based on the uptake, retention, and elution of crystal violet. Cells were plated at low density (0.5×10^3 cells) in 24-well plates (Becton Dickinson) and incubated for up to 8 days. At days 2, 4, 6, and 8, the cells were fixed with 1% glutaraldehyde (Sigma Chemical Co., St. Louis, MO) and stained with 0.5% crystal violet (Sigma) and washed as described [11]. Crystal violet dye was eluted with 0.5 ml of Sorenson's solution (9 mg of trisodium citrate in 305 ml of distilled H₂O, 195 ml of 0.1 N HCl, and 500 ml of 90% ethanol), and 100 μ l were transferred to a 96-well plate. In all experiments the absorbency of each well was measured by a Titertek Multiscan TCC/340 (Flow Laboratories, McLean, VA) at 560 nm, unless otherwise specified. Control experiments demonstrated that absorbance is directly proportional to the number of cells in each well. Experiments were performed in three wells per cell line. Experiments were repeated twice.

Measurement of Prostate-Specific Antigen (PSA)

Once tumors became measurable, blood samples for sequential PSA measurements were obtained by dorsal tail vein incision. Samples were collected in 75-mm microhematocrit capillary tubes and centrifuged, and the sera were stored at -20°C until further processing. PSA values in the serum were determined by a microparticle enzyme immunoassay (MEIA) for the quantitative measurement of PSA in an Abbott IMx

clinical analyzer (assays kindly provided by Abbott Laboratories, Abbott Park, IL).

For in vitro PSA measurement, cells were plated on Falcon plastic dishes (Becton Dickinson) and grown in T-medium to 80–100% confluence. After changing of the medium, PSA was measured in the supernatant 24 hr later as described above, and normalized to the number of cells in the dish. Experiments were repeated 3–7 times per cell line.

Cytogenetic Analysis

Cultures from B2–B5, fed 24 hr earlier with fresh medium, were exposed to Colcemid (final concentration, 0.02 $\mu\text{g/ml}$) for 40 min at 37°C . Cells from these cultures were dislodged by exposing them to 2 ml Hank's balanced salt solution containing 0.01% trypsin. The single-cell suspension in 5 ml of RPMI-1640 containing 10% FBS was centrifuged at 1,700 rpm for 5 min. After discarding the supernatant, the cell pellet was disturbed and exposed to a hypotonic solution (0.06 M KCl) for 15–20 min. at room temperature. After centrifugation (1,700 rpm for 5 min), cells were fixed in acetic acid:methanol (1:3, v/v) for 15 min and then washed three times in the fixative. Conventional air-drying chromosome preparations were made following routine laboratory techniques, as described elsewhere [12].

Invasiveness

To determine the invasiveness of the different cell lines, Costar 12-well plates (Costar, Cambridge, MA) having a polycarbonate filter insert (polyvinylpyrrolidone-free, 8- μm pore size) were coated with commercial Matrigel (Collaborative Biomedical Products, Bedford, MA) at a 1:3 dilution with serum-free T-medium and allowed to gel in a 37°C incubator for 30 min. LNCaP, C4-2, and B2–B5 cells were trypsinized to single-cell suspensions, and 2.5×10^3 cells in 0.5 ml of T-medium (5% FBS) were placed onto the Matrigel. One milliliter of T-medium (5% FBS) was placed in the lower compartment. Assays were incubated for 24 hr at 37°C with 95% air plus 5% CO₂. The filters on the side of the lower compartment were stained with crystal violet, and after washing, the remaining dye was wiped with a cotton swab and the dye on the swab was eluted with Sorensen's solution. Then the cells in the upper compartment were stained, washed, and eluted. The absorbance of each well was measured. Invasiveness was calculated as percent (%) absorbance of the lower compartment/absorbance of the upper and lower compartment. Experiments were performed in triplicate.

Intrinsic Tumorigenic Activity of the Bone Metastatic Cell Lines

Intrinsic anchorage-independent growth (soft agar colony formation) activity *in vitro* closely reflects the tumorigenicity of target epithelial cells [13]. Therefore, 2.5×10^3 LNCaP, C4-2, and B2-B5 cells were trypsinized to single-cell suspensions; the cells were resuspended in 0.3% agarose containing T-medium supplemented with 2% TCM and plated on a 12-well plate (Costar) containing 0.6% agarose as a bottom layer. The cover layer, consisting of T-medium and 2% TCM, was changed weekly. Colonies larger than 0.4 mm were scored 6 weeks after plating.

RNA Blot Analysis

LNCaP, C4, C4-2, and B2-B5 cells were cultured to 70–80% confluence; 48 hr prior to harvesting, cells were downshifted to serum-free conditions. Each cell line was then treated with 10^{-9} M dihydrotestosterone (DHT) for 48 hr. Cells remaining under serum-free conditions served as control for basal PSA mRNA expression. Total cellular RNA was extracted from cells by the RNeasy B[®] method (Qiagen, Crawfordsville, IN), a single-step purification protocol described by Chomczynski and Sacchi [14]. Equal amounts of RNA as determined by absorbance at 260 nm were subjected to RNA blot analysis by electrophoresis in a 0.9% agarose gel containing 2 M formaldehyde. RNAs were transferred by capillary blotting onto Zetaprobe[®] membrane (Bio-Rad, Richmond, CA), using $1 \times$ TAE (0.04 M Tris-acetate, 1 mM EDTA) buffer. RNAs were cross-linked to the membranes by ultraviolet exposure, using a Stratalinker[®] (Stratagene, La Jolla, CA) at 1,500 μ J, and membranes were prehybridized in hybridization buffer (10% dextran sulfate, 1% standard saline citrate, 1 M NaCl, and 20 μ g/ml salmon sperm DNA). The solution hybridization was performed by incubation at 65°C overnight by exposing the membranes to a 32 P-labeled PSA cDNA probe having a specific activity $>1 \times 10^8$ dpm/ μ g. After hybridization, the membranes were washed in $2 \times$ standard saline citrate at room temperature for 30 min, and were then washed under highly stringent conditions (2×30 min in $2 \times$ standard saline citrate/1% sodium dodecylsulfate, then 1×30 min $0.5 \times$ standard saline citrate/1% sodium dodecylsulfate) at 65°C. Autoradiograms were prepared by exposing Kodak X-Omat AR films to the membrane at -80°C with intensifying screens. Autoradiograms of RNA blot analysis for PSA were analyzed and PSA values were normalized to 18 S mRNA by means of ImageQuant[®] (Molecular Dynamics, Sunnyvale, CA).

Histomorphologic Characterization of Tumors

Specimens for routine histological examination were fixed in 4% paraformaldehyde and 5 mM MgCl_2 . Six-micron paraffin-embedded tumor sections were cut and stained with hematoxylin and eosin. Bone metastases were decalcified in 0.25 M EDTA.

RESULTS

In a previous study [6], we established an androgen-independent, tumorigenic, and bone metastatic cell line, C4-2, through a series of coinoculations of the human prostate cancer cell line LNCaP and the bone stromal cell line MS. In the present study, we focused on the characterization of the LNCaP cell sublines that metastasize to the bone and cell lines derived directly from those bone metastases. As shown in Figure 1, the AI C4-2 subline and the four AI bone metastasis-derived sublines, denoted as B2-B5, were derived from C4-2 tumors grown repeatedly in castrated male hosts, with the exception of B2, which was grown in an intact male host.

Cytogenetic analysis demonstrated that all four C4-2 bone metastasis-derived sublines (B2-B5) were of human origin [5,6]. The chromosome numbers in these C4-2 sublines varied between 80–89. Figure 2A–E shows typical G-banded karyotypes of the parental C4-2 and its bone metastasis-derived sublines, B2–B5. The cell lineage relationship was confirmed by common marker chromosomes, which were chromosomes 1, 2, 6, 10, 13, 15, and 16. Chromosome 8 had an 8p deletion in all sublines. B4 and B5 did not acquire any new marker chromosomes. The B2 subline acquired both stochastic deletion of the Y chromosome and the appearance of a new marker, a translocation between chromosomes 3q and 12q. B3 acquired two marker chromosomes: $m1 = i(7q)$ in some metaphases, and $m2 = \text{der}(11)t(11p+?)$. These markers appear to be associated with metastatic LNCaP sublines [5,6]. B5 is deleted for Y chromosome.

The growth curve of the parental LNCaP cell line *in vitro* was distinctly slower than that of its sublines C4-2, B2, B3, B4, and B5, as shown in Figure 3A. Previous studies showed that soft agarose colony-forming activity correlated well with *in vivo* tumorigenicity [3]. Results of this study, however, showed that anchorage-independent growth of C4-2 and B4, but not B2, B3, and B5 sublines, reflected accurately the *in vivo* growth potential of these bone metastasis-derived cell lines (Fig. 3B, Table I). Although there appear to be correlations between the latency of tumor growth, with B4 tumors growing faster than B2, B3, and B5, the frequencies of anchorage-independent

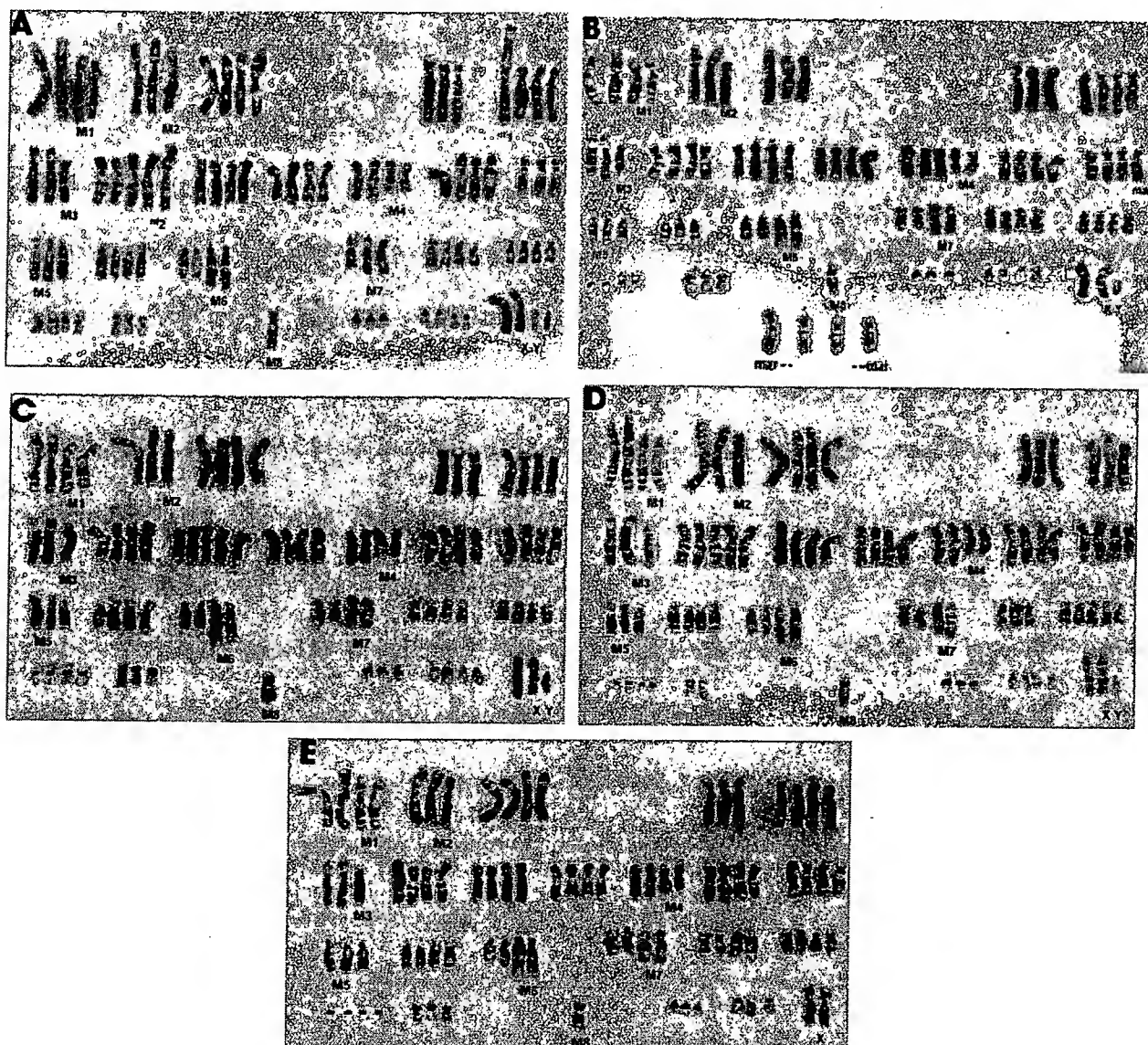


Fig. 2. Cytogenetic analysis: representative G-banded karyotypes for each of the cell lines, C4-2 (A), and C4-2 B2–C4-2 B5 (B–E), are displayed. Karyotype analysis confirms that cell lines are both human and derived from the C4-2 parental cell line, which was used to derive the bone metastatic variants.

growth of the latter were substantially lower than those of the parental LNCaP cells, which were shown to be nontumorigenic and nonmetastatic when inoculated subcutaneously in athymic hosts [3,5,6]. In comparison to the parental LNCaP cell line, all of the metastatic LNCaP cell sublines (C4-2, and B2–B5) presented a 2–3-fold higher invasiveness in a transwell Matrigel invasion assay (Fig. 3C).

RNA blot analysis indicates that all LNCaP sublines with a defined cell lineage relationship express mRNA for prostate-specific antigen (Fig. 4A,B). PSA mRNA expression can still be induced by dihydrotestosterone

(DHT) at physiologic concentrations, but to a lesser degree in the AI cell lines (Fig. 4A,B). Interestingly, androgen slightly suppressed PSA expression in the B3 cell line (Fig. 4B). Due to the unexpectedly low level of PSA mRNA in the B4 subline, we subcloned this line to determine whether this was clonal variation among B4 clones. Several single-cell clones of B4 (such as B4 (6-1); see Fig. 4A,B) were observed to have elevated PSA mRNA levels. Conversely, an additional observation was made when reinjecting the B3 cell line in vivo. A cell line isolated from a B3 tumor expressed high levels of PSA in vivo, but upon subcloning of a

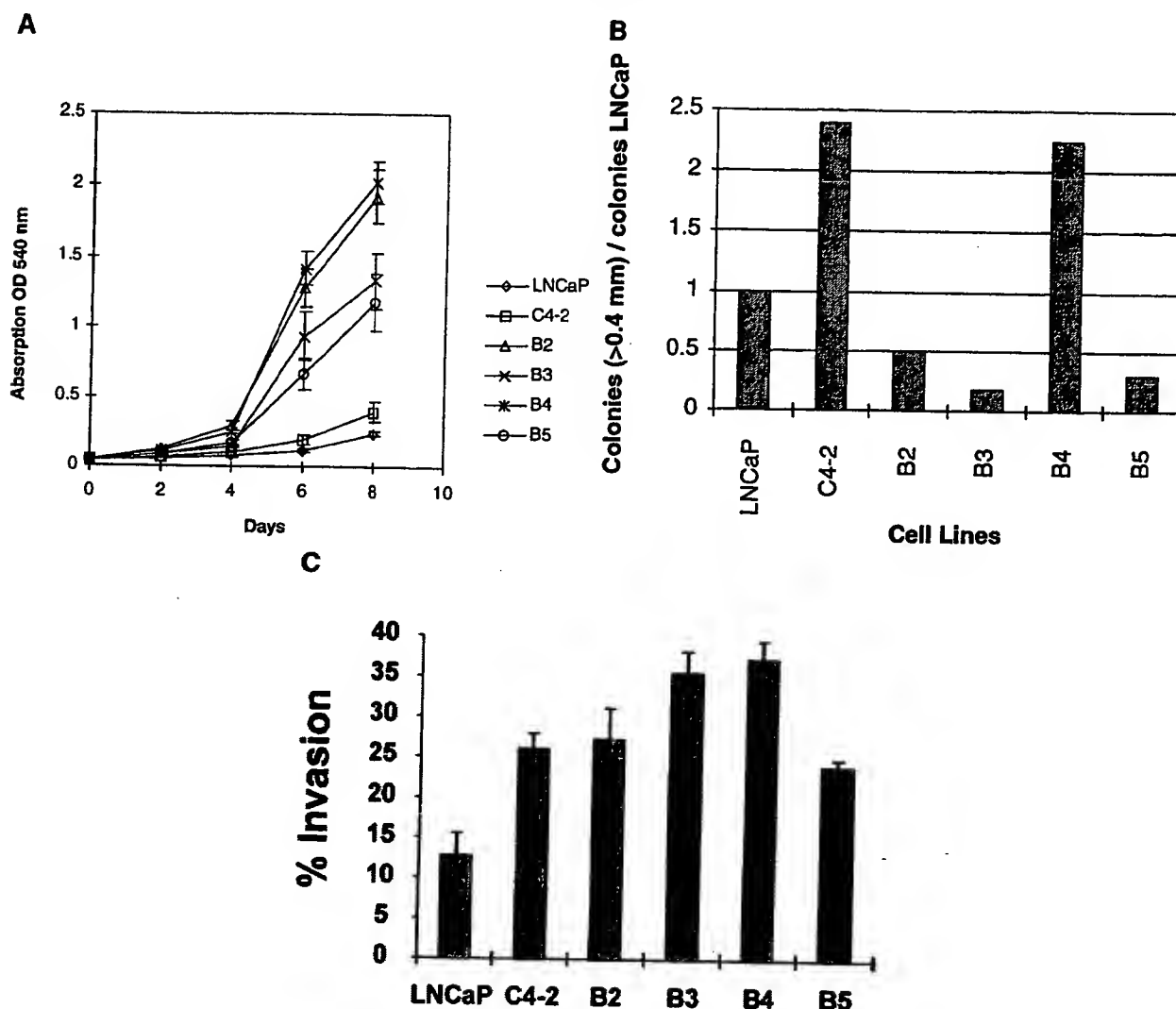


Fig. 3. In vitro assays. **A:** In vitro growth curves in 5% FCS assessed in a crystal violet growth assay. **B:** Anchorage-independent growth in a soft agar colony formation assay, normalized to LNCaP. **C:** Invasiveness of LNCaP and sublines in a Matrigel invasion assay expressed in percent of cells invading Matrigel.

TABLE I. Tumor Formation and Metastasis in Castrated Host by Subcutaneous Injection

Cell line	Tumor formation s.c.	Latency (median; range)	Paraplegia	Latency (median; range)
B2	4/4 (100%)	11 wks (10–12)	1/4 (25%)	19 wks (19)
B3	7/8 (87.5%)	11.2 wks (11–13)	3/8 (37.5%)	14.3 wks (13–16)
B4	4/4 (100%)	7.3 wks (5–11)	1/4 (25%)	5 wks (5)
B5	4/4 (100%)	10.7 wks (9–12)	1/4 (25%)	16 wks (16)

B3 tumor, the B3.1 subclone expressed substantially lower levels of PSA in vitro (Fig. 4A,B). These results suggest that the microenvironment of the tumor cells plays a key role in determining the steady-state levels of PSA expression in prostate tumor cells.

RNA blot analysis for the human androgen receptor consistently demonstrated a 9.4-kb transcript for the human androgen receptor in all cell lines (data not shown).

To determine if the C4-2 sublines were metastatic in vivo, we compared both the tumorigenicity and the

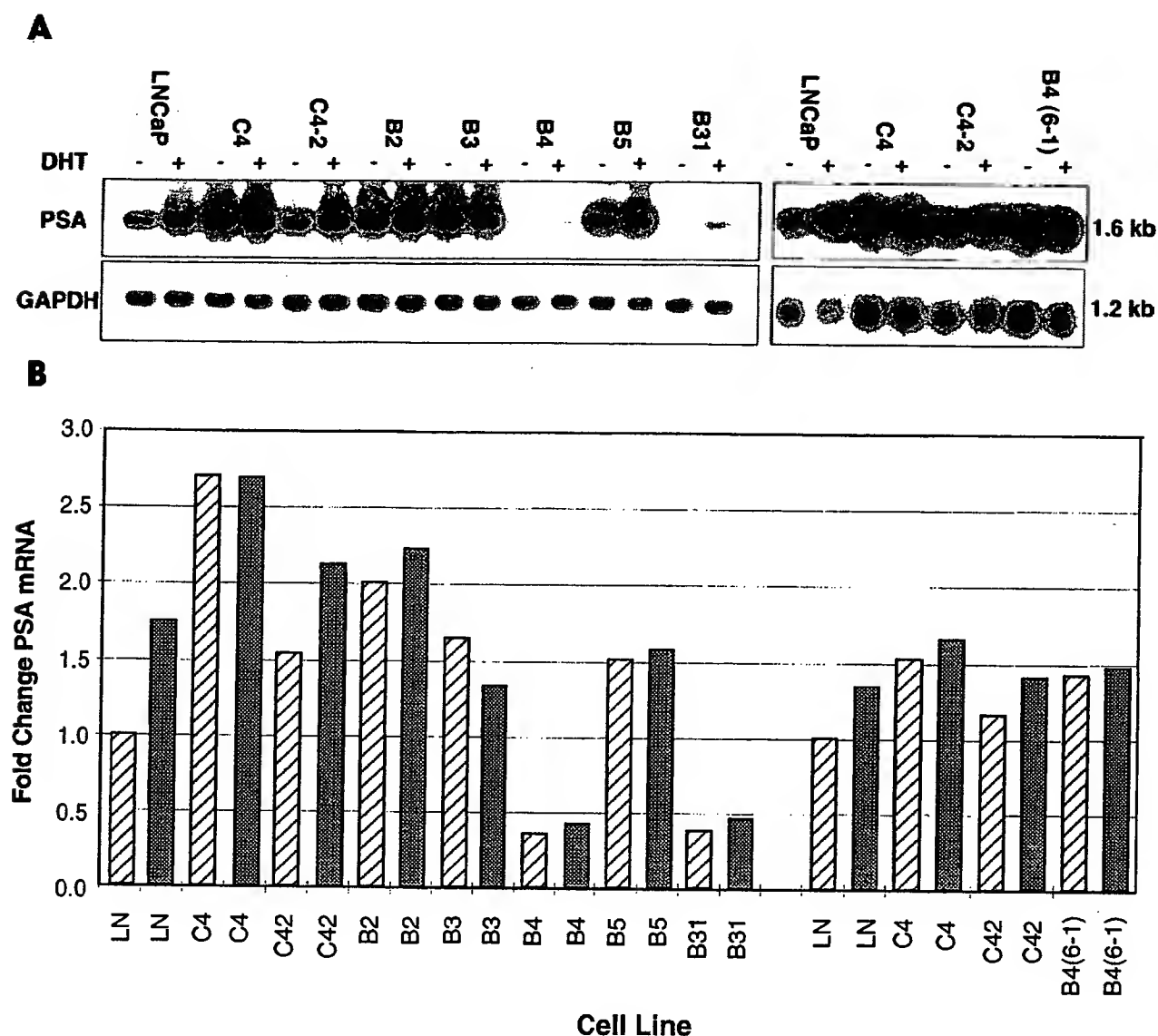


Fig. 4. Regulation of PSA expression in the LNCaP cell lineage model by dihydrotestosterone. Cells were exposed to dihydrotestosterone (DHT) at 10^{-8} M for 48 hr under serum-free culture conditions. **A, left:** Androgen-induced PSA mRNA expression in parental LNCaP and its lineage-derived sublines. Intrinsic PSA was lower in the B4-derived subline from an early passage culture. DHT induced an increased PSA mRNA in the androgen-dependent rather than the androgen-independent LNCaP sublines. Androgen-independent C4-2 and its derivative bone sublines responded to a lesser extent than the parental LNCaP to DHT. **Right:** Responsiveness of PSA mRNA to DHT in the LNCaP subline, B4 variant 6-1. Note that this variant has a much higher basal level expression of PSA; it remained unresponsive to DHT. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as loading control. **B:** Determination of the fold change in PSA mRNA expression with (+) and without (-) treatment with DHT. All data are expressed as fold change PSA/GAPDH and are normalized to LNCaP without DHT treatment.

metastatic potential of these C4-2 sublines. Whereas the incidence of tumor formation in mice inoculated with 1.0×10^6 cells of the LNCaP subline C4-2 was affected substantially by the site of injection (100% orthotopic vs. 1.5% s.c.) [6], the tumorigenicity of 1.0×10^6 cells each of the four bone metastasis-derived sublines, denoted B2-B5, was not influenced by the site of

injection; subcutaneous reinoculation of the bone metastasis-derived cell lines B2-B5 resulted in rapid tumor growth, with incidences ranging from 87.5–100% (Table I). Bone metastasis-derived cell lines grew tumors more rapidly in vivo than the C4-2 cell line (5–13 weeks as compared to 9–14 weeks), and a fraction of the animals (25–37.5%) developed paraplegia faster

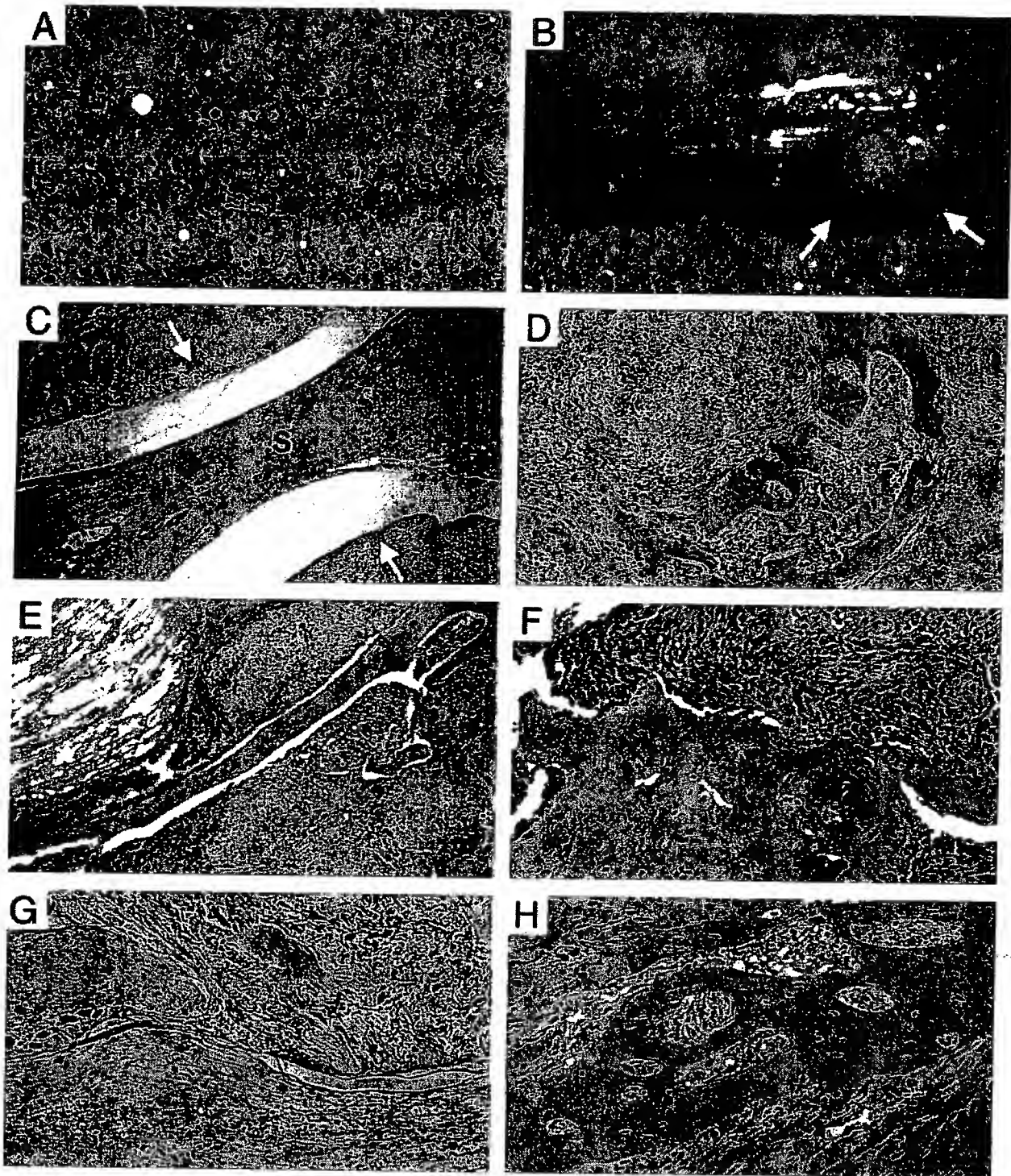


Fig. 5. Macroscopic and histological characterization of LNCaP sublines. **A:** Primary tumor of the B4 subline. Note the presence of multiple mitotic figures. **B:** Dissected spine of a paraplegic animal due to osseous metastasis of the B4 subline. Arrows indicate enlarged spinal metastasis. **C:** Longitudinal section of a spine with osseous metastasis and compression of the spinal cord. Arrows indicate the encroaching bone metastasis resulting in spinal compression. **D:** Spinal metastasis resulting from the B2 subline. Note the poor organization and high degree of dysplasia found in this tumor. **E:** Spinal metastasis derived from a B3 subline. Note a poorly organized carcinoma encroaching on the spinal cord. **F:** High magnification of a B3-derived spinal metastasis. This high-magnification view clearly shows osteoblastic response and bone remodeling by the B3 subline. **G:** Low-power view of a B4 spinal metastasis. Note poorly organized carcinoma with remodeled and newly deposited osteoid encroaching upon the spinal cord. **H:** High-power view, showing bone remodeling from a B5 subline-derived spinal metastasis. Newly deposited osteoid contains carcinomatous elements (A, F, H, $\times 62.5$; C, D, E, G, $\times 31.25$).

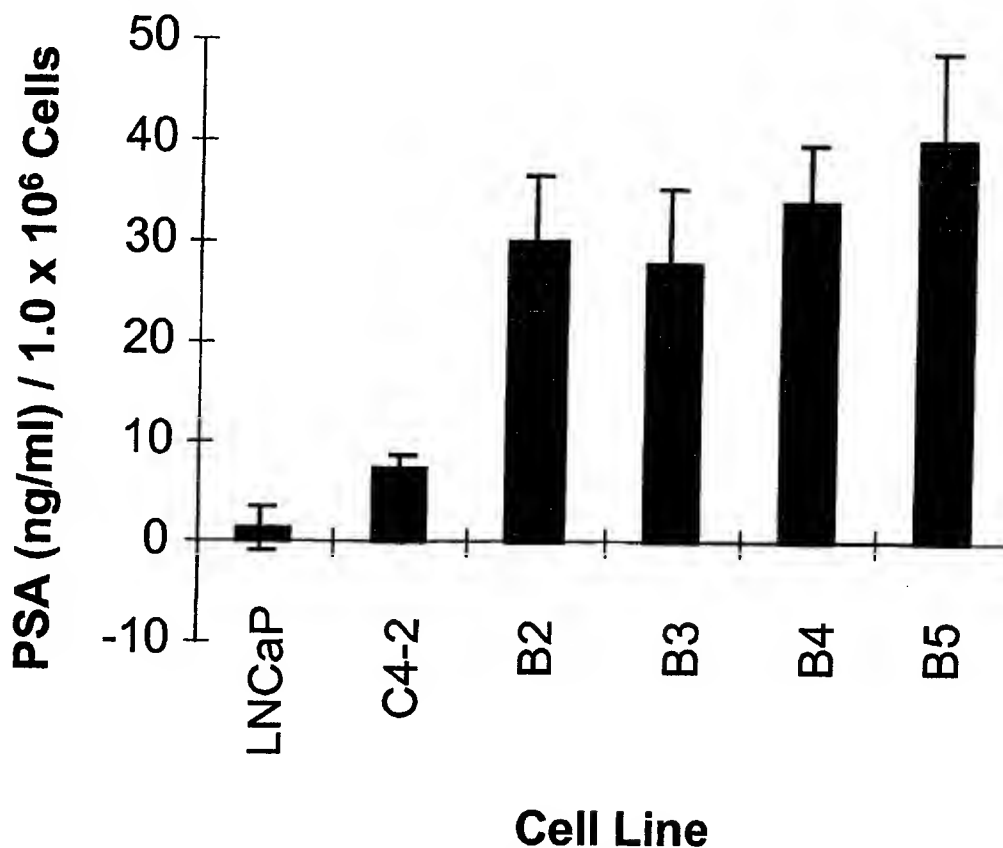


Fig. 6. Secretion of PSA protein measured in ng/ml of LNCaP and lineage-derived sublines in vitro. Data were expressed as mean (\pm standard deviation) PSA protein secreted by the different cell lines per 1.0×10^6 cells.

(5–19 weeks) than those animals inoculated with C4-2 cells (mean, 29 weeks; range, 17–43 weeks; see Thalmann et al. [6]). Histomorphologically, the primary tumors were poorly differentiated (Fig. 5A).

One animal inoculated subcutaneously with B4 cells developed osseous metastasis as early as 5 weeks. Overall, the bone metastasis-derived cell lines had a bone metastasis incidence of 33.3% (range, 25–37.5%). Seven of 20 (35%) animals developed cachexia prior to osseous metastasis and had to be sacrificed.

Bone metastases (Fig. 5B) induced a vast osteoblastic reaction (Fig. 5D–H), with compression of the spinal cord and subsequent paraplegia (Fig. 5C). Figure 5D–H depicts a panel of osseous metastases (B2–B5). The osteoblastic tumor deposits had an increased basal metabolism, and hence elevated uptake and sequestration of ^{99m}Tc -methylene diphosphate in whole-body sagittal imaging bone scans, as shown previously [6] (data not shown).

PSA expression was generally high initially in animals carrying LNCaP subline-derived tumors, but PSA became greatly depressed when tumors were maintained in hosts for longer periods (>5 months, data not shown). Animals injected with C4-2 or bone

metastasis-derived LNCaP sublines developing osseous metastases and paraplegia tended to secrete lower levels of PSA in vivo than animals who did not develop bone lesions. Interestingly, this was reversed in vitro. As demonstrated in Figure 6, the bone metastasis-derived cell lines secreted up to 20-fold more PSA per million cells into the medium than the parental LNCaP cell line. The B4 cell line expressed large amounts of PSA protein in vitro, despite low mRNA levels.

The phenotypic characteristics of the LNCaP model of prostate cancer progression is summarized in Table II and indicates that by passaging cells through hosts, the phenotype can be altered permanently in terms of tumorigenicity, metastatic potential, cytogenetic status, and biologic behavior.

DISCUSSION

The process of metastasis is a cascade of linked sequential steps involving complex genetic and epigenetic interaction [4,15]. To gain further insight into the molecular events that lead to cancer metastasis and to establish useful experimental models of prostate can-

TABLE II. Phenotypic Characterization of Parental LNCaP, Androgen-Independent Sublines C4 and C4-2, and Bone Metastasis-Derived Cell Lines B2, B3, B4, and B5

Cell line	No. of in vivo passages	Androgen-dependent	PSA production	Tumorigenicity		Metastasis to the skeleton
				Male	Castrated host	
LNCaP	0	Yes	Yes	No	No	No
C4	1	No	Yes	Yes	No	No
C4-2	2	No	Yes	Yes	Yes	Yes
B2	3	No	Yes	Yes	Yes	Yes
B3	3	No	Yes	Yes	Yes	Yes
B4	3	No	Yes	Yes	Yes	Yes
B5	3	No	Yes	Yes	Yes	Yes

cer metastasis, a number of approaches have been implemented. For example, genetic manipulation of prostate epithelial cells either through oncogene transfection [16–18] or transgenic mice using tissue-specific promoters [19–21] has resulted in low or no incidence of bone metastasis, with little evidence of osteoblastic reaction. Recognizing the importance of the tumor-host microenvironment interaction in dictating tumor behavior in vivo [4,22,23], we have derived human prostate cancer cell lines from tumor xenografts or their metastases by subculturing tumor cells from chimeric xenografts [3–6,8], and rat prostate cancer epithelial and stromal cell lines by altering the host microenvironment [9]. These efforts resulted in obtaining prostate tumor cells with defined tumorigenic and metastatic potential.

In the development of an in vivo mouse model of human prostate cancer metastasis, we observed that a marginally tumorigenic human prostate cancer cell line, LNCaP, can be induced to acquire and/or adapt to these changes, resulting in AI progression, with the resulting cell acquiring tumorigenic and osseous metastatic phenotypes when grown under androgen-depleted conditions with inductive prostate or bone stromal cell lines in vivo [5,6,8]. These findings exemplify the key role of stromal cells and the hormonal status of the host in directing cell-cell interaction and dictating the changes leading to the subsequent expression of androgen-independent and metastatic phenotypes. The present communication documents the genotypic and phenotypic alterations of human prostate cancer cell lines derived from spontaneous experimental bone metastases of LNCaP lineage-derived cell sublines. These LNCaP sublines have acquired diverse tumorigenic and metastatic potential.

The LNCaP model of prostate cancer progression mimics the clinical features of human disease: 1) Androgen-dependent (defined as prostate tumor or cells capable of growing subcutaneously and secreting PSA

only in intact male hosts when inoculated with supporting stromal cells or extracellular matrix) LNCaP cells progress to become androgen-independent (defined as cells capable of forming PSA-secreting prostate tumors in castrated male hosts without supporting stromal cells or extracellular matrix) upon interaction with bone stromal cells in castrated hosts, resulting in the derivation of lineage-related LNCaP sublines C4-2, and B2–B5. 2) Androgen-independent LNCaP sublines synthesize and secrete PSA in an AI manner. However, a reduced level of PSA expression by androgen-induction was observed. The LNCaP derivative sublines express androgen receptor, and this receptor is functional and can be stimulated by physiologic and superphysiologic concentrations of exogenous androgens. 3) When grown orthotopically, LNCaP sublines induced localized prostate cancer and growth, invading the seminal vesicles and metastasizing to the lymph nodes and the bone, where they invoked a marked osteoblastic reaction of the skeleton. Animals subsequently developed signs of paraplegia and cachexia followed by death [6]. The behavioral patterns of invasion, migration, and dissemination are commonly observed in men with clinical prostate cancer. 4) Analysis by means of comparative genomic hybridization (CGH) [24] confirmed the cell-lineage relationship between the parental LNCaP and its sublines. Although the regions of chromosomal losses or gains are not in complete concordance using either CGH or conventional cytogenetics, it was noted that marked chromosomal changes occurred in LNCaP bone metastasis-derived sublines, as defined by both conventional (Fig. 2) and CGH [24] techniques. Remarkably, 15 of the 16 genetic changes documented in the C4-2 human prostate cancer progression cell line [24] were reported in cytogenetic studies of clinical specimens of human prostate cancer [25–30]. These results suggest that the LNCaP human prostate cancer progression model has the unique advantage of

closely mimicking the phenotypic and genotypic changes often found in clinical human prostate cancer.

PSA mRNA expression is elevated in the LNCaP sublines (Fig. 4A,B). The fold of androgen induction of PSA mRNA decreases with the acquisition of androgen-independence (Fig. 4B). Interestingly, the B4 cell line, which expresses PSA on the protein level in vitro and in vivo, expressed low amounts of PSA mRNA in vitro. Single B4 cell clones were then expanded and showed PSA expression comparable to that of the other bone metastasis-derived cell lines, indicating clonal diversity. This is also supported by the fact that a B3-derived subline, expressing high PSA in vivo, demonstrated low levels of PSA mRNA expression in the derivative B3.1 subline (Fig. 4A,B). In other cases, however, PSA was expressed initially in the tumor xenografts (e.g., C4-2 tumors), but the levels of PSA decreased markedly as tumors were maintained in the castrated hosts for longer periods until bone metastasis was detected. We attribute this to downregulation of PSA expression by soluble factors secreted by bone stromal cells. Koenen et al. (unpublished observations) showed that PSA promoter-reporter expression in LNCaP and C4-2 cells is depressed by the conditioned media obtained from bone stromal cell lines. Finally, all LNCaP sublines expressed PSA at the protein level, with the level of PSA expression positively correlated with the osseous metastatic potential of the derivative sublines (Fig. 6).

Of the four bone metastasis-derived cell lines, only B4, which was derived from a castrated host that was injected with C4-2 cells orthotopically (presumably cellular interaction occurs between C4-2 cells and the host mouse prostate stroma), showed increased tumorigenicity and rapid development of bone metastases when subsequently injected s.c. in athymic castrated male mice. The other bone metastasis-derived C4-2 sublines (B2, B3, and B5) originated from subcutaneous administration of C4-2 cells in intact or castrated hosts. These observations suggest that the stromal-epithelial interaction (with stromal cells derived either from the skin or the prostate) under androgen-depleted conditions might subselect cell clones with a more malignant phenotype. A growing body of experimental evidence supports the concept that androgen deprivation enhances the development of androgen-independent growth and the acquisition of metastatic potential by prostate cancer cells. In the Noble [31] and Dunning [32] rat models, androgen-independent and metastatic prostate cancer cell clones were selected from tumors maintained in androgen-deprived male hosts. In the androgen-dependent Shionogi mouse mammary tumor model [33], castration produced an androgen-independent subclone. In

the present LNCaP progression model of human prostate cancer, we have demonstrated that such selection/adaptation may occur in tumors maintained in castrated hosts. These results may have profound implications when considering the natural course of prostate cancer androgen-independent progression in androgen-deprived hosts. After hormonal therapy, prostate cancer frequently progresses to an androgen-refractory state. A subset of these patients with androgen-independent disease, treated with maximal androgen blockade using nonsteroidal antiandrogens, may progress and could benefit from a withdrawal of the nonsteroidal antiandrogens such as flutamide and bicalutamide [34]. This may be a consequence of prolonged androgen-deprivation. Based on results obtained from tissue culture and animal models, it was suggested that long-term culture of prostate cancer cells in an androgen-deprived environment [35,36] results in the development of androgen suprasensitivity or androgen receptor mutations. It is conceivable that intermittent androgen suppression, which is currently being evaluated in clinical trials, might depress the emergence of androgen-independent clones from the stem-cell populations within prostate tumors [37].

Overall, these findings illustrate the importance of the host and tumor environment on the biologic behavior of tumor cells. Although vascular anatomy [38,39] may account in part for the selectivity of prostate cancer for the axial skeleton, considerable experimental evidence [3,5,6,8,13,40] has substantiated that local factors, such as bone fibroblasts, extracellular matrix, and growth factors, play an important role in tumor progression and metastasis, thus supporting Paget's "seed in fertile soil" theory [41].

In summary, we established an osseous metastatic model of human prostate cancer. Evidence suggests that androgen-deprivation may facilitate androgen-independent progression and acquisition of metastatic potential by prostate cancer cells in a nonrandom manner. We have characterized the molecular, biochemical, and cytogenetic characteristics of osseous metastasis-derived LNCaP sublines in vitro and have correlated these findings with the tumorigenicity and metastatic potential of these cells in vivo. This model of human prostate cancer progression and metastasis closely mimics the genetic and pathological processes of cancer growth and progression in men.

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